

Oxidative Modification of Neurofilament-L by Copper-catalyzed Reaction

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Neurofilament-L (NF-L) is a major element of neuronal cytoskeletons and known to be important for neuronal survival *in vivo*. Since oxidative stress might play a critical role in the pathogenesis of neurodegenerative diseases, we investigated the role of copper and peroxide in the modification of NF-L. When disassembled NF-L was incubated with copper ion and hydrogen peroxide, then the aggregation of protein was proportional to copper and hydrogen peroxide concentrations. Dityrosine crosslink formation was obtained in copper-mediated NF-L aggregates. The copper-mediated modification of NF-L was significantly inhibited by thiol antioxidants, N-acetylcysteine, glutathione, and thiourea. A thioflavin-T binding assay was performed to determine whether the copper/H₂O₂ system-induced *in vitro* aggregation of NF-L displays amyloid-like characteristics. The aggregate of NF-L displayed thioflavin T reactivity, which was reminiscent of amyloid. This study suggests that copper-mediated NF-L modification might be closely related to oxidative reactions which may play a critical role in neurodegenerative diseases.

Keywords: Copper, Hydrogen peroxide, Modification, Neurofilament-L

Introduction

Neurofilaments give axons their structural integrity and define axonal diameter (Hoffman *et al.*, 1987; Brady *et al.*, 1993). Neurofilaments are composed of three subunits. These subunits are identified as light (NF-L), medium (NF-M), and heavy (NF-H), which assemble in a 6 : 2 : 1 ratio to form long macromolecular filaments (Nixon *et al.*, 1986; Nixon *et al.*, 1992). Consequently, NF-L is more abundant than the other two subunits in neurons. NF-L is capable of homologous

assembly, whereas NF-M and NF-H are not component to assemble in the absence of NF-L (Cohlberg *et al.*, 1995). Abnormal accumulation of neurofilaments in neurons is one of the pathological hallmarks of various neurodegenerative disorders. In patients with Parkinson's disease, Lewy bodies (LBs) are cytoplasmic inclusions that are consistently present and have the greatest frequency in neurons of the substantia nigra. Also, locus ceruleus NF proteins have been identified immunohistochemically as major protein components of the LB filament (Forno, 1986; Gidd *et al.*, 1989). Peroxynitrite may nitrate tyrosine residues of NF-L, thereby altering NF assembly and causing neurofilament accumulation in neurons (Crow *et al.*, 1997).

Reactive oxygen species (ROS) from metabolic or adventitious reduction of O₂ are associated with the etiology of human diseases (Eum and Kang, 1999; Halliwell and Gutteridge, 1999). The oxidation of cellular proteins has been described under many pathological conditions (Ames *et al.*, 1993; Berlett *et al.*, 1997). Cellular metabolism generates oxygen species, such as hydrogen peroxide, hydroxyl radical, and superoxide anion. Transition metals, such as iron and copper, react with hydrogen peroxide to produce hydroxyl radical through Fenton-type reaction and can induce the oxidative modification of DNA, protein, and lipids (Halliwell and Gutteridge, 1999). During exposure to hydrogen peroxide, many proteins with metal binding sites will probably be susceptible to oxidative damage, and free metal ions could be released. The oxidative modification of Cu,Zn-superoxide dismutase by hydrogen peroxide induced the release of copper ions (Kang and Kim, 1997). Free copper that is released from a certain Cu,Zn-SOD mutant in familial amyotrophic lateral sclerosis (Yim *et al.*, 1996; Eum and Kang, 1999) and copper that is reduced by the amyloid precursor protein in Alzheimer's disease (Multhaup *et al.*, 1996) were suggested to be a part of etiology for these diseases. Therefore, the reaction of copper with hydrogen peroxide may be associated with the abnormal modification of neurofilaments, which may be involved in the pathogenesis of neurodegenerative disorders.

In this study, we investigated whether the copper-catalyzed oxidation system is involved in the aggregation of NF-L. Our

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results revealed that the copper-catalyzed reaction induced the aggregation of NF-L and the formation of amyloid-like fibrils. These results suggest that the copper-catalyzed oxidation system may be involved in the oxidative stress-induced aggregation of NF-L in neurodegenerative diseases.

Materials and Methods

Expression and purification of neurofilament-L A full-length cDNA clone of mouse NF-L in a pET-3d vector, a generous gift from Dr. Beckman (University of Alabama), was transfected into *E. coli* (BL21). The protein expression was performed as previously described (Ozden *et al.*, 2002). Bacteria were grown in a Luria broth that was supplemented with 1 mM isopropyl β -D-thiogalactopyranoside, beginning at an OD 600 nm reading of 0.8. Incubation was at 37°C for 3 h. Bacteria were harvested by centrifugation ($4,000 \times g$ for 10 min at 4°C) and resuspended in a standard buffer (50 mM Mes, 170 mM NaCl, 1 mM DTT, pH 6.25). The cells were lysed with a French press at a pressure of 20,000 psi and centrifuged at $8,000 \times g$ for 15 min at 4°C. The supernatant was incubated for 3 h at 37°C, and then was centrifuged at $100,000 \times g$ for 20 min at 25°C. The pellets that contained the aggregated NF-L proteins were washed twice with a standard buffer before they were dissolved in a 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. The column was washed with the urea buffer. The column was eluted with a linear 25-500 mM phosphate gradient in the urea buffer and the NF-L was eluted between 300 and 360 mM phosphate. These fractions were pooled and either used directly or stored at -70°C for later experiments. The protein concentration was determined by the BCA method (Smith *et al.*, 1985).

Oxidation of protein Oxidative modification of NF-L (0.8 mg/ml) was carried out by incubation of the protein with CuSO_4 and H_2O_2 in 10 mM potassium phosphate buffer (pH 7.5) at 37°C. After the incubation, the mixtures were then placed into a Ultrafree-MC filter and centrifuged at 13,000 rpm for 1 h, then washed with Chelex 100-treated water and centrifuged for 1 h at the same speed. This was repeated four times. The filtrate was dried by a freeze drier and dissolved with a phosphate buffer.

Analysis of modified protein After treatment with various concentrations of Cu^{2+} and H_2O_2 for various periods of time, the reaction mixture samples were diluted with a 5x loading-sample buffer (62.5 mM Tris, 10% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.01% bromophenol blue) and heated in boiling water for 10 min before loading onto SDS-PAGE (Treerattrakool *et al.*, 2002). An aliquot of each sample was subjected to SDS-PAGE, as described by Laemmli (Laemmli, 1970), using a 12% acrylamide slab gel. The gels were stained with 0.15% Coomassie Brilliant Blue R-250.

Detection of o,o-dityrosine The reactions for the detection of o,o-dityrosine were carried out with NF-L (0.8 mg/ml), CuSO_4 , and H_2O_2 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 Spectrofluorometer SMF 25 (Bio-Tek Instruments, Winooski, USA).

Thioflavin-T binding assay Thioflavin-T staining was performed to determine whether the copper-catalyzed oxidation reaction-induced NF-L aggregates displayed amyloid-like characteristics. Briefly, NF-L reacted with CuSO_4 and H_2O_2 in 10 mM potassium phosphate (pH 7.4). The sample mixture (15 μl) was added to 2 ml of 10 μM thioflavin-T in 50 mM Glycine/NaOH, pH 8.5, and analyzed with a fluorescence spectrometer. The sample fluorescence was monitored in the 460-520 region (excitation at 450 nm) using Spectrofluorometer SMF 25 (Bio-Tek Instruments).

Results

An SDS-PAGE analysis showed that NF-L remained intact after incubation with CuSO_4 or H_2O_2 alone (Fig. 1A). In contrast, the staining intensity for the original protein was reduced, and new high molecular weight material was visualized at the stacking/separating gel interface when NF-L was incubated with both Cu^{2+} and H_2O_2 . These results suggest that both Cu^{2+} and H_2O_2 were required for the aggregation of NF-L. The aggregation of NF-L became apparent at 50 μM CuSO_4 ; the aggregation increased up to 500 μM CuSO_4 (Fig. 1B). The aggregation of NF-L was also increased in a H_2O_2 concentration-dependent manner.

It has been reported that the o,o-dityrosine crosslink formation between the dityrosine residues may play a part in the formation of the oxidative covalent protein crosslink (Stadtman *et al.*, 1993). We investigated the formation of o,o-dityrosine during the $\text{Cu}^{2+}/\text{H}_2\text{O}_2$ system-mediated NF-L aggregation by measuring the 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 of 300 μM CuSO_4 for 0 and 7 h at 37°C. As the reactions proceeded, the emission peak at 410 nm increased, due to the formation of o,o-dityrosine crosslinks (Fig. 2). Oxidative protein crosslinks are produced by several means, such as direct interactions between carbon-centered radical derivatives of amino acids. Our result suggested that the tyrosine-tyrosine crosslink formation might participate in the $\text{Cu}^{2+}/\text{H}_2\text{O}_2$ system-mediated NF-L aggregation.

The effect of antioxidant molecules on the aggregation of NF-L by the $\text{Cu}^{2+}/\text{H}_2\text{O}_2$ system was studied. The aggregation of NF-L by the $\text{Cu}^{2+}/\text{H}_2\text{O}_2$ system was significantly suppressed in the presence of N-acetylcysteine, glutathione, and thiourea (Fig. 3). These results suggest that hydroxyl radical might play a critical role in the aggregation of NF-L by the $\text{Cu}^{2+}/\text{H}_2\text{O}_2$ system.

Neurofilament is a major component of aggregates that form amyloid-like fibrils in Lewy bodies (LBs) of Parkinson's disease (PD) and senile plaques of Alzheimer's disease (AD). A thioflavin-T binding assay was performed to determine

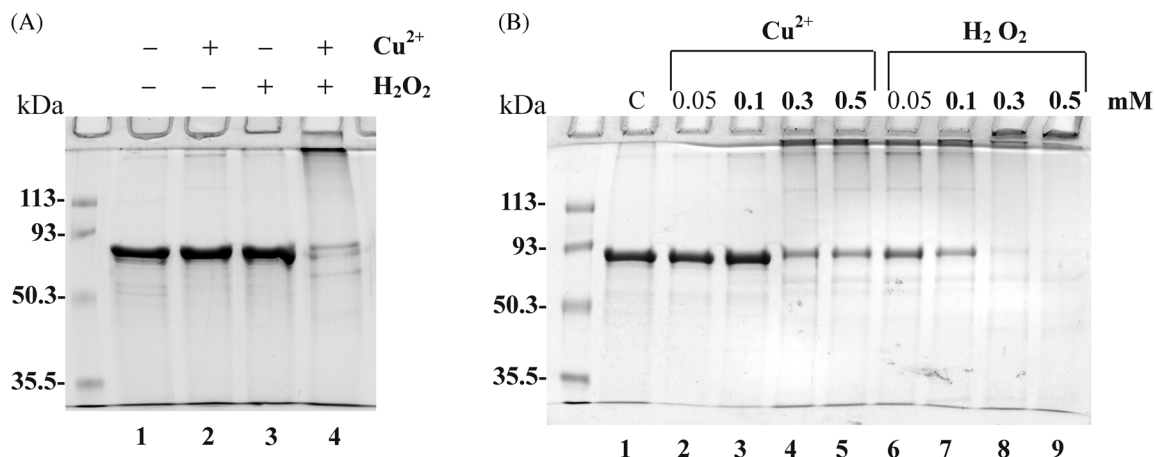


Fig. 1. Modification of NF-L by copper-mediated oxidation reaction. (A) NF-L (0.25 mg/ml) was incubated with Cu^{2+} and H_2O_2 in 10 mM phosphate buffer (pH 7.4) at 37°C for 4 h. Lane 1, NF-L control; lane 2, NF-L + 0.3 mM Cu^{2+} ; lane 3, NF-L + 0.1 mM H_2O_2 ; lane 4, NF-L + 0.3 mM Cu^{2+} + 0.1 mM H_2O_2 . (B) NF-L (0.8 mg/ml) was incubated with various concentrations of Cu^{2+} and H_2O_2 in 10 mM phosphate buffer (pH 7.4) at 37°C for 4 h. Lane 1, NF-L control; lane 2, 0.05 mM Cu^{2+} + 0.1 mM H_2O_2 ; lane 3, 0.1 mM Cu^{2+} + 0.1 mM H_2O_2 ; lane 4, 0.3 mM Cu^{2+} + 0.1 mM H_2O_2 ; lane 5, 0.5 mM Cu^{2+} + 0.1 mM H_2O_2 ; lane 6, 0.3 mM Cu^{2+} + 0.05 mM H_2O_2 ; lane 7, 0.3 mM Cu^{2+} + 0.1 mM H_2O_2 ; lane 8, 0.3 mM Cu^{2+} + 0.3 mM H_2O_2 ; lane 9, 0.3 mM Cu^{2+} + 0.5 mM H_2O_2 . The molecular weight marker positions (kDa) are indicated on the left.

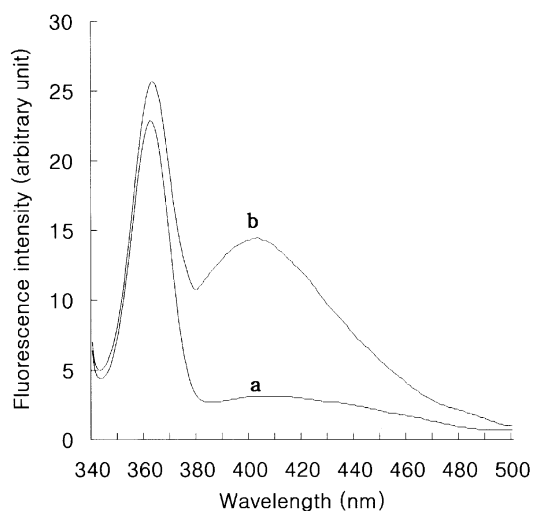


Fig. 2. Fluorescence spectra of copper-mediated NF-L modification. The fluorescence spectra of the formation of dityrosine were observed when NF-L (0.8 mg/ml) was incubated with Cu^{2+} and H_2O_2 . (a) NF-L control, (b) NF-L with 0.3 mM Cu^{2+} and 0.1 mM H_2O_2 .

whether the $\text{Cu}^{2+}/\text{H}_2\text{O}_2$ system-induced *in vitro* aggregation of NF-L displayed amyloid-like characteristics. As shown in Figure 4, strong reactivity was obtained with NF-L, indicating that the $\text{Cu}^{2+}/\text{H}_2\text{O}_2$ system may induce the formation of amyloid-like fibrils of NF-L.

Discussion

NF-L, a major structural protein that is important for the

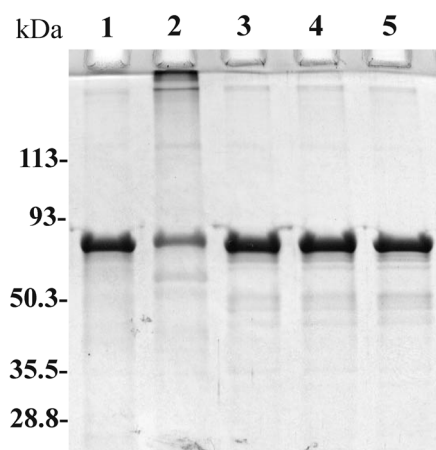


Fig. 3. Effect of thiol antioxidants on copper-mediated NF-L modification. NF-L (0.8 mg/ml) was incubated with 0.3 mM Cu^{2+} and 0.1 mM H_2O_2 in 10 mM phosphate buffer (pH 7.4) at 37°C for 4 h in the presence of thiol antioxidants. Lane 1, NF-L control; lanes 2 and 5, no addition; lane 3, 20 mM N-acetylcysteine; lane 4, 20 mM glutathione; lane 5, 20 mM thiourea. The molecular weight marker positions (kDa) are indicated on the left.

survival of motor neurons, was modified by oxidative stress. Neurofilaments are susceptible to oxidation, in part because they are among the most abundant proteins in a cell. Previous studies suggested that oxidative stress might play a critical role in the pathogenesis of PD (Halliwell, 1989; Youdim *et al.*, 1989; Jenner *et al.*, 1996). Trace metals (such as copper and iron, which are present in the biological system) react with hydrogen peroxide and then produce the hydroxyl radicals. This reactive oxygen might lead to the cross-linking and

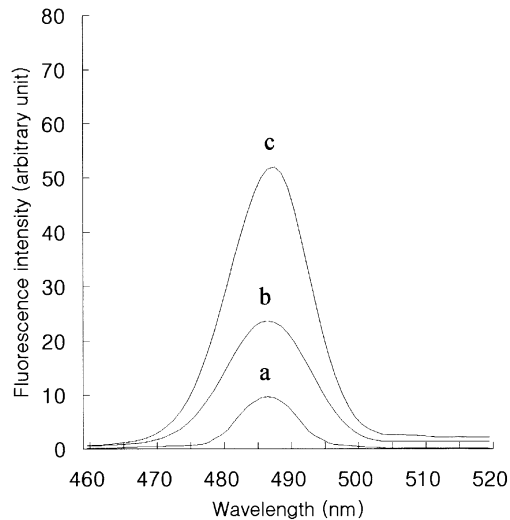


Fig. 4. Fluorescence spectra of thioflavin-T-bound NF-L aggregate. The fluorescence spectra of the product by the reaction of thioflavin-T with NF-L aggregate were observed when NF-L (0.8 mg/ml) was incubated with Cu^{2+} and H_2O_2 . (a) 0.3 mM Cu^{2+} and 1 mM H_2O_2 , (b) NF-L control, (c) NF-L with 0.3 mM Cu^{2+} and 1 mM H_2O_2 .

aggregation of amyloidogenic molecules (Markesbery *et al.*, 1999). The cleavage of the metalloproteins by oxidative damage may lead to increases in the levels of metal ions in some biological cells (O'Connell *et al.*, 1987). The releasing of copper ions from Cu,Zn-SOD could be induced by a free radical-generating function of the enzyme (Kang and Kim, 1997). Since copper promotes NF-L self-aggregation, it is possible that the aberrant accumulation of copper ion might act as a risk factor for neurodegenerative diseases. It has been reported that the copper concentration significantly increased in the cerebrospinal-fluid of PD patients (Pall *et al.*, 1987). Recently, it was reported that the cerebrospinal-fluid copper concentration increased 2.2-fold in AD patients (Multhaup *et al.*, 1996). These results suggest that the iron or copper-catalyzed oxidative reaction might contribute to the pathogenesis of neurodegenerative diseases.

Many antioxidant molecules have been proposed for the therapeutic agents of neurodegenerative diseases (Edwards *et al.*, 2002; Floyd *et al.*, 2002; Nagano *et al.*, 2003). Our results show that thiol antioxidants, such as N-acetylcysteine, glutathione, and thiourea, could protect the aggregation of NF-L by the copper-catalyzed oxidation system. 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. Thiol antioxidants are very efficient copper chelating agents (Jimenez *et al.*, 2000; Zhu *et al.*, 2002). These compounds might be able to bind Cu^{2+} and prevent some Cu^{2+} -dependent radical reactions. N-Acetylcysteine is a scavenger of hydroxyl radicals, as determined by a pulse radiolysis experiment (Felix

et al., 1996). Therefore, it can be assumed that thiol antioxidants may protect the aggregation of NF-L by the copper-catalyzed oxidation system through the combination of scavenging of $\cdot\text{OH}$ radicals and the chelation of copper ion.

In conclusion, the present results suggest that the aggregation of NF-L was induced by the copper-catalyzed oxidation system that involved $\cdot\text{OH}$ generation from H_2O_2 . Therefore, the copper-catalyzed oxidation system-mediated NF-L aggregation might be involved in the pathogenesis of neurodegenerative diseases.

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