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Acrolein, the toxic endogenous aldehyde, induces neurofilament-L aggregation

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Acrolein is a highly reactive by product of lipid peroxidation and individuals with neurodegenerative disorders have been shown to contain elevated concentrations of this molecule in the brain. In the present study, we examined the pattern of neurofilament-L (NF-L) modification elicited by acrolein. When NF-L was incubated with acrolein, protein aggregation occurred in a acrolein concentration-dependent manner. Exposure of NF-L to acrolein also led to the generation of protein carbonyl compounds. Through the addition of free radical scavengers we observed a significant decrease in acrolein-mediated NF-L aggregation. These results indicate that free radicals may be involved in the modification of NF-L by acrolein. In addition, dityrosine cross-link formation was observed in acrolein-mediated NF-L aggregates and these aggregates displayed thioflavin T reactivity, reminiscent of amyloid. This study suggests that acrolein-mediated NF-L aggregation might be closely related to oxidative reactions, thus these reactions may play a critical role in neurodegenerative diseases. [BMB reports 2008; 41(9): 635-639]

INTRODUCTION

Oxidative stress has been implicated in a range of neurodegenerative disorders, including Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). The hallmarks of oxidative stress include, lipid peroxidation, protein oxidation and DNA oxidation. Lipid peroxidation, which results from free radical damage to polyunsaturated fatty acids, also generates cytotoxic aldehydes like malondialdehyde (1), 4-hydroxynonenal (HNE) (2), and acrolein (3). These aldehydes exert cytotoxicity and genotoxicity by adducting cellular neucleophilic groups found on proteins, lipids, and nucleic acid (4). Previous studies have demonstrated that acrolein is more toxic than formaldehyde, acetaldehyde and HNE (5-10). Acrolein has been shown to be associated with proteins that were detected in neurofibrillary tangles and distrophic neuritis surrounding senile plaques in AD (11). In a recent study increased concentrations of acrolein was found in the brains of patients with AD (7).

Neurofilaments are a major cytoskeletal component of neurons. The three mammalian neurofilament subunits, NF-L (~68 kDa), NF-M (~145 kDa), and NF-H (~200 kDa), are believed to form heterodimers consisting of NF-L in combination with either NF-M or NF-H (12). Abnormal accumulation of NF in neurons has been associated with neurodegenerative disorders such as AD (13), PD (14) and ALS (15-17). It has been reported that overexpressing human NF-L in mice led to the severe loss of neurons in the parietal cortex and ventrobasal thalamus with age (18). In addition, an approximate four-fold increase in NF-L expression has been shown to lead to an ALS-like pathology in mice (19). In these studies, degeneration and neuron loss, which resembles the pathology of ALS, was observed in transgenic mice that overexpressed mouse NF-L (19). The transgenic mouse showed intensive NF aggregation in all compartments of the neurons with depleted rough endoplasmic reticulum. This study confirmed that overexpressing of NF-L and NF aggregation in mice can directly cause abnormality and degeneration of motor neuron.

In the present study, the effect of acrolein on the modification of NF-L was investigated. Our results revealed that the aggregation of NF-L was induced by acrolein through the generation of free radicals. Furthermore, the formation of amyloid-like fibrils in acrolein-mediated NF-L aggregates was obtained using the thioflavin T binding method. These results suggest that the endogenous aldehyde, acrolein, may be involved in the oxidative stress-induced aggregation of NF-L in neurodegenerative diseases.

RESULTS AND DISCUSSION

Acrolein exists in the environment as a ubiquitous pollutant that is generated as a by-product of overheated organic materials. *In vivo*, acrolein is formed in the metal-catalyzed oxidation of polyunsaturated fatty acids including arachidonic acid (20). The toxicity of acrolein may be augmented by its free radical-generating function in cells. Since the levels of free radicals were reported to increase in patients with degenerative disorders (21, 22), the oxidative modification of NF-L may have pathological significance. To investigate acrolein-mediated modification of NF-L, reaction mixtures contain-

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ing these components were subjected to SDS-PAGE analysis. As shown in Fig. 1(A), there was an acrolein concentration-dependent increase in the formation of protein aggregates. When NF-L was incubated with 2 mM acrolein, most of the protein migrated to the top of the gel. Since it has been shown that protein oxidation leads to the conversion of some amino acid residues to carbonyl derivatives, the carbonyl content of these samples was measured (23). The carbonyl content of protein can be measured using a phenylhydrazine formation reaction. The method for detecting carbonyl-containing proteins employs derivatization with 2,4-dinitrophenyl hydrazine (DNPH) followed by analysis with anti-DNP sera. Result obtained from the immunoblotting analysis of acrolein-treated NF-L was shown in Fig. 1 (B). In this analysis, carbonyl compounds were detected in the major bands and protein aggregates. These results suggest that free radicals might be involved in the modification of NF-L by acrolein.

The participation of free radicals in the modification of NF-L by acrolein was determined by examining the effects of free radical scavengers on NF-L aggregation during the reaction of NF-L with acrolein. *N*-Acetyl-$_L$-cysteine is a derivative of the sulfur-containing amino acid cysteine and an intermediary in the conversion of cysteine to reduced glutathione (GSH), which has an intracellular concentration of 1-20 mM in human cells (24). In addition, the thiol group confers antioxidant effects and it has been widely used as an antioxidant in revealing the role of free radicals in preconditioning induced tissue protection (25, 26). Therefore, *N*-acetyl-$_L$-cysteine and GSH were used as a model free radical scavenger to determine the effects of free radicals on acrolein-induced NF-L aggregation. In these experiments, the modification of NF-L was significantly suppressed in the presence of *N*-acetyl-$_L$-cysteine and GSH (Fig. 2). These results suggest that acrolein may lead to the generation of free radical.

It has been reported that o,o′-dityrosine crosslink formation between dityrosine residues may play a role in the formation of oxidative covalent protein crosslinks (27). We investigated the formation of o,o′-dityrosine during the acrolein-mediated NF-L aggregation by exciting the reactions at 325 nm and then measuring the fluorescence emission spectrum between 340 and 500 nm. The NF-L−acrolein reactions were carried out in various concentrations of acrolein for 24 h at 37°C. As the concentration of acrolein was increased, the formation of o,o′-dityrosine crosslink also increased (Fig. 3). Oxidative protein crosslinking can be produced through several means, such as the direct interactions between carbon-centered radical derivatives of amino acids. Our result suggested that the tyrosine-tyrosine crosslink formation may participate in acrolein-mediated NF-L aggregation.

Neurofilaments are a major component of aggregates that form amyloid-like fibrils in Lewy bodies (LBs) of PD and senile plaques of AD. To determine whether the acrolein-induced *in vitro* aggregation of NF-L display amyloid-like characteristics, thioflavin-T binding assays were performed. As shown in Fig. 4, a strong reactivity was observed with NF-L, indicating that acro-

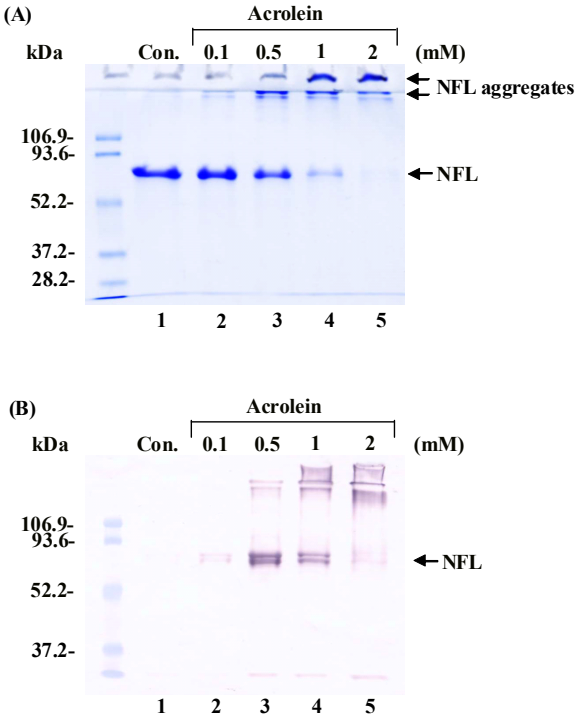

Fig. 1. Modification of NF-L by acrolein and detection of carbonyl compounds. (A) NF-L (0.15 mg/ml) was incubated with various concentrations of acrolein in 10 mM phosphate buffer (pH 7.4) at 37°C for 24 h. Lane 1, NF-L control; lane 2-5, 0.1, 0.5, 1 and 2 mM acrolein, respectively. (B) Reaction mixtures contain NF-L and various concentrations of acrolein, DNPH was derivatized as described under Materials and Methods. DNPH-derivatized proteins were subjected to SDS-PAGE for immunoblot with anti-DNP sera. The positions of molecular weight markers (kDa) are indicated on the left.

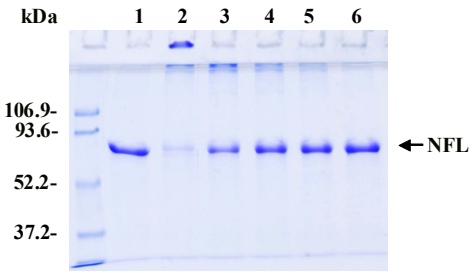

Fig. 2. Effect of free radical scavengers on the modification of NF-L by acrolein. NF-L (0.15 mg/ml) was incubated with 1 mM acrolein in 10 mM phosphate buffer (pH 7.4) at 37°C for 24 h in the presence of free radical scavengers. Lane 1, NF-L control; lane 2, no addition of free radical scavengers; lane 3, 1 mM N-acetyl cysteine; lane 4, 10 mM N-acetyl cysteine; lane 5, 1 mM glutathione; lane 6, 10 mM glutathione.

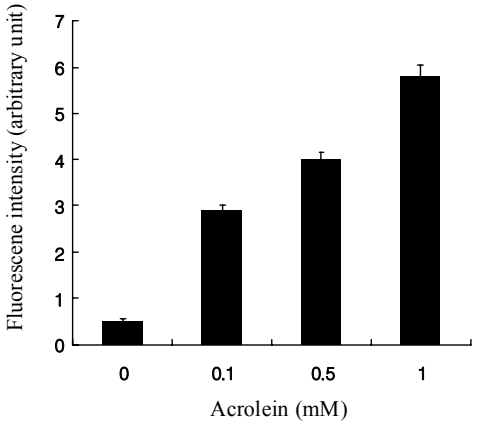

Fig. 3. Fluorescence spectra of acrolein-mediated NF-L modification. The fluorescence spectra of the formation of dityrosine was observed when NF-L (0.15 mg/ml) was incubated with various concentrations of acrolein.

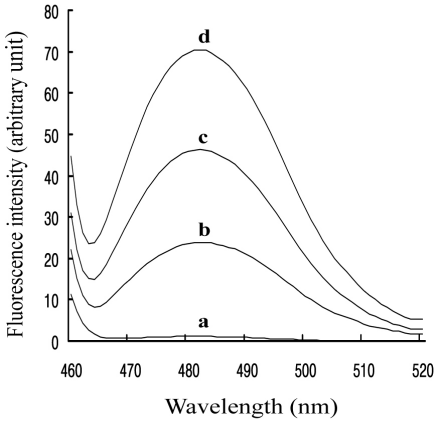

Fig. 4. Fluorescence spectra of thioflavin T-bound NFL aggregates. The fluorescence spectra of the product by the reaction of thioflavin T with NFL aggregates was observed when NF-L (0.15 mg/ml) was incubated with various concentrations of acrolein. (a) NF-L control, (b) NF-L with 0.1 mM acrolein (c) NF-L with 0.5 mM acrolein (d) NF-L with 1 mM acrolein.

lein may induce the formation of amyloid-like fibrils of NF-L. Cytoskeleton disruption is a prominent feature and secondary event following oxidative damage in AD (28). Neurofibrillary tangles, the hallmark of AD, is composed of abnormally modified NFs and other cytoskeleton proteins.

In conclusion, the results of the present study suggest that the aggregation of NF-L was induced by acrolein and this aggregation appears to involve free radical generation. Furthermore, our results indicate that acrolein-mediated NF-L aggregation might be associated with the pathogenesis of degenerative disorders.

MATERIALS AND METHODS

Materials

Acrolein, *N*-acetyl-L-cysteine, glutathione, and thioflavin-T 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 protein

A full-length cDNA clone of mouse NF-L in a pET-3d vector was a generous gift from Dr. Beckman (University of Alabama) and transfected into *E.coli* (BL21). Protein expression was performed as previously described (29). Bacteria were grown in Luria broth supplemented with 1 mM isopropyl β-D-thiogalactopyranoside beginning at an OD 600 nm reading of 0.8. Bacteria were incubated at 37°C for 3 h, then harvested by centrifugation (4,000 g for 10 min at 4°C), and resuspended in a standard buffer (50 mM MES, 170 mM NaCl, 1 mM DDT, pH 6.25). The cells were lysed 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 centrifuged at 100,000 g for 20 min at 25°C. The pellets containing the aggregated NF-L proteins were washed twice with a 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 DDT). The sample was then loaded onto a DEAE-Sepharose column and the column was washed with urea buffer. The column was eluted with a linear 25-500 mM phosphate gradient in urea buffer and 1 ml NF-L eluted between 300 and 360 mM phosphate. These fractions were pooled and either used directly or stored at −70°C for use in future experiments.

Protein modification

Protein concentration was determined by the BCA method (30). Modification of NF-L (0.15 mg/ml) was carried out by incubation of the protein in a 10 mM potassium phosphate buffer (pH 7.4) both in the presence and absence of acrolein at 37°C. After completion of the reaction, the mixtures were then placed into Microcon filter (Amicon) and centrifuged at 13,000 rpm for 1 h to remove acrolein. The mixture was then washed with Chelex 100 treated water and centrifuged for 1 h at 13,000 rpm to further remove acrolein. This process was repeated four times. The filtrate was dried by a freeze dryer and dissolved with 10 mM potassium phosphate buffer (pH 7.4). The free radical experiments were performed by preincubation of the protein with the free radical scavengers for 5 min at room temperature followed by reacting the protein/free radical mixture with acrolein for 24 h at 37°C. The unreacted reagents wer washed using Microcon filter (Amicon).

Analysis of NF-L modification

NF-L (0.15 mg/ml), in 10 mM potassium phosphate buffer, pH 7.4, was incubated at 37°C for 24 h with different concen-

trations of acrolein in a total volume of 20 μl. The samples were treated with 7 μl of 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 as described by Laemmli (31), using a 18% acrylamide slab gel. The gels were stained with 0.15% Coomassie Brilliant Blue R-250.

Detection of protein carbonyl compound

The carbonyl content of proteins was determined by immunoblotting with anti-DNP antibodies as described elsewhere (23). Both native and oxidized proteins were incubated with 20 mM 2,4-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 in an 18% polyacrylamide gel, the proteins were transferred onto a nitrocellulose sheet and then probed with rabbit anti-DNP sera, at a dilution of 1:1000. Rabbit anti-DNP was detected using alkaline phosphatase-labelled goat anti rabbit IgG with the BCIP/NBT detection system (Bio-Rad).

Detection of o,o′-dityrosine

Detection of o,o′-dityrosine were carried with NF-L (0.15 mg/ml) and acrolein in 10 mM potassium phosphate (pH 7.4). 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).

Thioflavin T binding assay

Since Acrolein-induced NF-L aggregates displayed amyloid-like characteristics, thioflavin T staining was performed. Briefly, NF-L was reacted with acrolein in 10 mM potassium phosphate (pH 7.4). A sample mixture (15 μl) was added to 2 ml of 10 μM thioflavin T in 50 mM Glycine/NaOH, at pH 8. The fluorescent emission spectrum of the sample was monitored in the 460-520 region (excitation, 450 nm) using Spectrofluorometer SMF 25 (Bio-Tek Instruments).

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