Protection by Histidine Dipeptides against Acrolein-induced Neurofilament-L Aggregation

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The endogenous dipeptides, carnosine and related compounds, are the naturally occurring dipeptides with multiple neuroprotective properties. We have examined the protective effects of carnosine, homocarnosine and anserine on the aggregation of neurofilament-L (NF-L) induced by neurotoxin, acrolein. When NF-L was incubated with acrolein in the presence of carnosine, homocarnosine or anserine, protein aggregation was inhibited in a concentration-dependent manner. These compounds inhibited the formation of protein carbonyl compounds and dityrosine in acrolein-mediated NF-L aggregates. The aggregates of NF-L displayed thioflavin T reactivity, reminiscent of amyloid. This thioflavin T reactivity was inhibited by carnosine and related compounds. This effect was associated with decreased formation of oxidatively modified proteins. Our results suggested that carnosine and related compounds might have protective effects to brain proteins under pathophysiological conditions leading to degenerative damage such as neurodegenerative disorders.

Key Words : Carnosine, Acrolein, Aggregation, Neurofilament-L

Introduction

Acrolein occurs in the environment as ubiquitous pollutant that is generated as a by-product of overheated organic materials. *In vivo*, acrolein informed in the metal-catalyzed oxidation of polyunsaturated fatty acids including arachidonic acid.[1] Acrolein is the strongest electrophile among the reactive aldehydes and shows the highest reactivity with neutrophiles including cysteine, histidine and lysine.[2] It has been reported that acrolein preferentially react with lysine residues of tau,[1] and that acrolein adducts were detected in neurofibrillary tangles and dystrophic neutrites surrounding senile plaques in Alzheimer disease (AD).[3]

Carnosine (β-alanyl-L-histidine) and related compounds anserine (β-alanyl-1-methyl-L-histidine) and homocarnosine (γ-amino-butyryl-L-histidine) are naturally occurring dipeptides present in the muscle and brain tissues of human and other vertebrates in relatively high concentrations (1-20 mM).[4,5] *In vitro* and *in vivo* studies have shown that carnosine can exert neuroprotective effects through various mechanisms such as cytosolic buffering abilities, antioxidant activity, and metal ion-chelating properties.[6-9]

Neurofilaments are the most abundant neuron-specific intermediate filaments[10] and represent a major element of the neuronal cytoskeleton. 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.[11] Abnormal accumulation of NF in neurons is associated with neurodegenerative disorders.[12-16] Previous studies have shown that the reactive aldehyde adducts of neurofilaments were detected in the brain and peripheral nerve of human and animals.[17-19]

In the present study, the effects of carnosine and related compounds on acrolein-mediated NF-L modification were investigated. Present results revealed that carnosine, homocarnosine and anserine inhibited NF-L aggregation induced by acrolein. These compounds also inhibited the formation of dityrosine and amyloid-like fibrils in acrolein-mediated NF-L aggregates.

Materials and Methods

Materials. Acrolein, carnosine, homocarnosine, anserine and thioflavin-T and 2,4-dinitrophenyl hydrazine (DNPH) 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, a generous gift from Dr. Beckman (University of Alabama) transfected into *E. coli* (BL21). Protein expression was performed as previously described.[20] Bacteria were grown in Luria broth supplemented with 1 mM isopropyl β-D-thiogalactopyranoside beginning at an OD 600 nm reading of 0.8. Incubation was at 37 ^{o}C for 3 h. Bacteria were harvested by centrifugation (4,000 g for 10 min at 4 ^{o}C), resuspended in 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 ^{o}C. The supernatant was incubated for 3 h at 37 ^{o}C and then was centrifuged at 100,000 × g for 20 min at 25 ^{o}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 DDT). The sample was 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 10 mL NF-L eluted between 300 and 360 mM

phosphate. These fractions were pooled and either used directly or stored at −70 °C for later experiments.

Protein modification. Protein concentration was determined by previously described.[21] Modification of NF-L (0.15 mg/mL) was carried out by incubation of the protein in 10 mM potassium phosphate buffer (pH 7.4) both in the presence and absence of acrolein at 37 °C. After incubation of the reaction mixtures, 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 same speed to further remove acrolein. This was repeated four times. The filtrate was dried by freeze dryer and dissolved with 10 mM potassium phosphate buffer (pH 7.4). The protection by carnosine and related compounds was performed by preincubation of the protein in the presence of carnosine and related compounds.

Analysis of NF-L modification. The reaction mixtures were treated with 7 μL of sample buffer (0.25 M Tris, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, 0.01% bromophenolblue) and were boiled at 100 °C for 5 min before electrophoresis.[22] Each sample was subjected to SDS-PAGE as described by Laemmli,[23] using a 18% acrylamide slab gel. The gels were stained with 0.15% Coomassie Brilliant Blue R-250.

Determination of protein carbonyl compound. The carbonyl content of proteins was determined by spectrophotometric assay as described elsewhere.[24] Both native and oxidized proteins were incubated with 10 mM 2,4-DNPH in 2.5 M HCl at room temperature for 1 h. After incubation, 20% TCA(w/v) solution was added to the sample and the tubes are left in ice bucket for 10 min and centrifuged for 5 min in a tabletop centrifuge to collect the protein precipitates. The supernatants are discarded. Next another wash is performed using 10% TCA, and protein pellets are broken mechanically with the aid of pipette tip. Finally, the pellets are washed 3 times with ethanol-ethyl acetate (1:1) (v/v) to remove the free DNPH. The final precipitates are dissolved in 2 mL of 6 M guanidine hydrochloride solution and are left for 10 min at 37 °C with general vortex mixing. Carbonyl content was calculated from the absorbance (370 nm) using an absorption coefficient e of 22,000 M^{-1} cm^{-1}.

Detection of *o,o'*-dityrosine. The reactions for the 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 (exitation, 325 nm) using Spectrofluorometer SMF 25 (Bio-Tek Instruments).

Thioflavin T binding assay. To determine whether acrolein-mediated NF-L aggregates displayed amyloid-like characteristics, thioflavin T staining was performed. Briefly, NF-L reacted with acrolein in 10 mM potassium phosphate (pH 7.4). Sample mixture (15 μL) was added to 2 mL of 10 mM thioflavin T in 50 mM Glycine/NaOH, pH 8.5 and analyzed with a fluorescence spectrometer (Bio-Tek Instruments). The fluorescence of the sample was monitored in the 410-480 region (exitation, 450 nm).

Results and Discussion

The toxicity of acrolein may be augmented by its free radical-generating function in cells.[25] Since the level of free radicals was reported to be increase in degenerative disorder patients,[26,27] the oxidative modification of NF-L by acrolein might have pathological significance. In the present study, we investigated the protective role of carnosine and related compounds in acrolein-mediated NF-L modification. When NF-L was incubated with 1 mM acrolein, most of the protein was at the top of the gel (Fig. 1A, B and C, lane 2). Carnosine, homocarnosine and anserine inhibited the aggregation of NF-L (Fig. 1A, B and C, lane 3-6). It has been shown that protein oxidation leads to the conversion of some amino acid residues to carbonyl derivatives.[28] The carbonyl content of protein can be measured using a phenylhydrazine formation

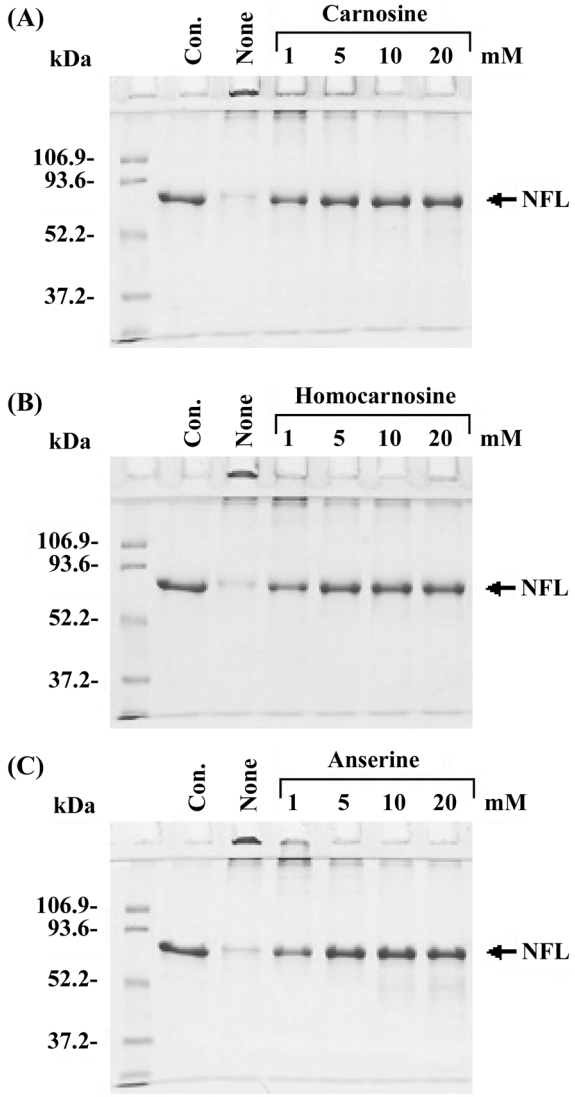

Figure 1. Effects of carnosine and related compounds on the aggregation of NF-L by acrolein. NF-L (0.15 mg/mL) was incubated with 1 mM acrolein in the presence of various concentrations of carnosine (A), homocarnosine (B) and anserine (C). The samples were alnalyzed by 12% SDS-PAGE. The positions of molecular weight markers (kDa) are indicated on the left.

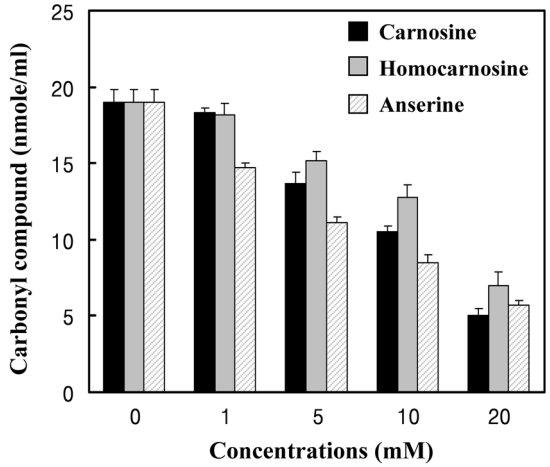

Figure 2. Effects of carnosine and related compounds on the formation of carbonyl compounds in acrolein-mediated NF-L aggregates. NF-L (0.15 mg/mL) was incubated with 1 mM acrolein in the presence of various concentrations of carnosine and related compounds. Reaction mixtures contain NF-L and various concentration of acrolein were derivatized DNPH as described under Materials and Methods. DNPH-derivatized proteins were determined by analysis with spectrophotometer.

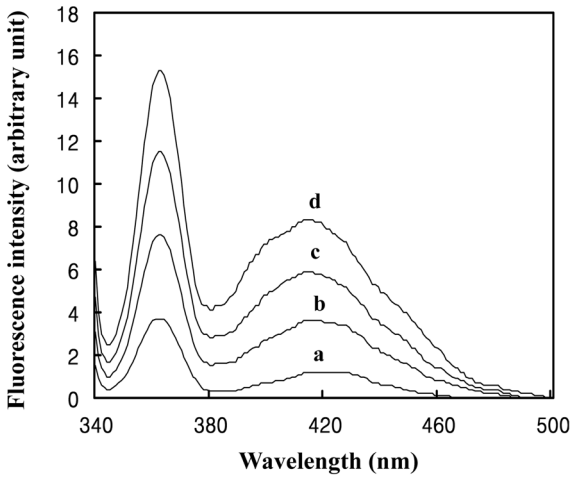

Figure 3. Formation of dityrosine in acrolein-mediated NF-L modification. The fluorescence spectra of the formation of dityrosine was observed when NF-L (0.15 mg/mL) was incubated with a various concentrations of acrolein. (a) NF-L control, (b) NF-L with 0.1 mM acrolein (c) NF-L with 0.5 mM acrolein (d) NF-L with 1 mM acrolein.

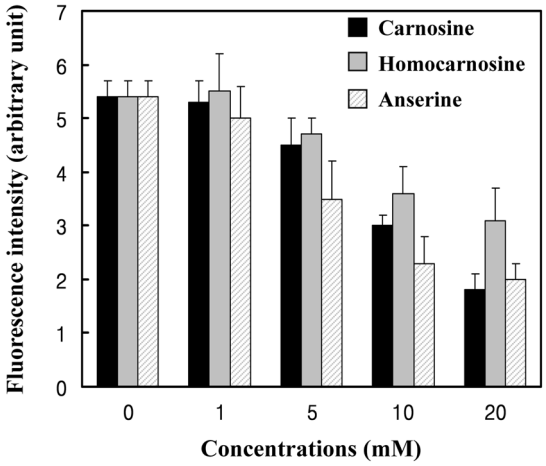

Figure 4. Effects of carnosine and related compounds on the formation of dityrosine in acrolein-mediated NF-L aggregates. NF-L (0.15 mg/mL) was incubated with 1 mM acrolein in the presence of various concentrations of carnosine, homocarnosine and anserine.

reaction. The method for detecting carbonyl-containing proteins employs derivatization with 2,4-DNPH followed by analysis with spectrophotometer.[24] Inhibition of carnosine, homocarnosine and anseine against the formation of carbonyl compounds in acrolein-treated NF-L was shown in Figure 2. Protein crosslinking is a consequence of oxidation that induces formation carbonyl groups. Carnosine can react with carbonyl groups and thereby modulate their oligomerization and deleterious interaction with other polypeptides.[29] Therefore, histidine-dipeptides should be explored as exogenous agents able to detoxify carbonyl compounds.

Previous study suggested that *o,o'*-dityrosine crosslink formation between dityrosine residues might play a part in the formation of oxidative covalent protein crosslink.[30] We investigated the formation of *o,o'*-dityrosine during the acrolein-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 various concentration of acrolein for 6 h at 37 ^{o}C. As the concentration of acrolein was increased, the formation of *o,o'*-dityrosine crosslink was increased (Fig. 3). Oxidative protein crosslink is produced through 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 acrolein-mediated NF-L aggregation. Carnosine, homocarnosine and anseine significantly inhibited the formation of dityrosine in acrolein-treated NF-L (Fig. 4). The imidazolium group of histidine or carnosine stabilizes adducts formed at the primary amino group and may play an important role for an anticrosslinking agent.[31]

Neurofilament proteins have been implicated in the pathogenesis of several neurodegenerative disorders including amyotrophic lateral sclerosis, AD and Parkinson disease (PD). The common feature in all these disorders is the slow accumulation of neurofilament aggregates in neuronal cells accompanied by cell death. Of the three subunits, NF-L seems to play the most important role in neurofilament assembly, since it is the only neurofilament protein capable of organizing filaments by it self.[32] Neurofibrillary tangles as the hallmark of AD is composed of abnormally modified NFs and other cytoskeleton proteins. The histological dye thioflavin-T is widely used for the detection of amyloid fibrils.[33,34] In the presence of fibrils, thioflavin-T gives rise to enhanced fluorescence emission at 482 nm, whereas unbound thioflavin-T is essentially non-fluorescent at this wavelength. To determine whether the acrolein-induced *in vitro* aggregation of NF-L displays amyloid-like characteristics, thioflavin-T binding assay was performed. As shown

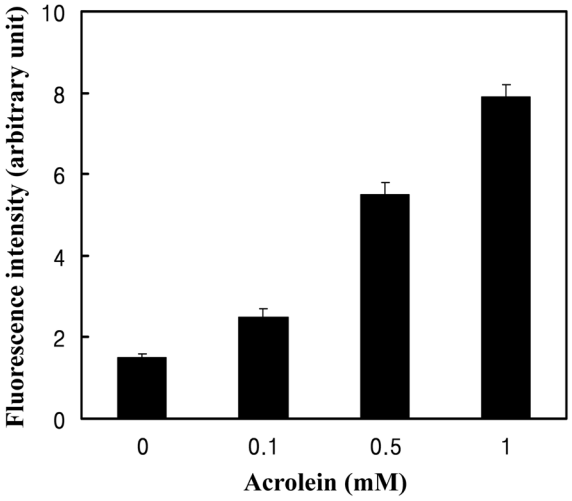

Figure 5. Fluorescence spectra of thioflavin T-bound NF-L aggregates. The fluorescence spectra of the product by the reaction of thioflavin T with NF-L aggregate was observed when NF-L (0.15 mg/mL) was incubated with a various concentrations of acrolein.

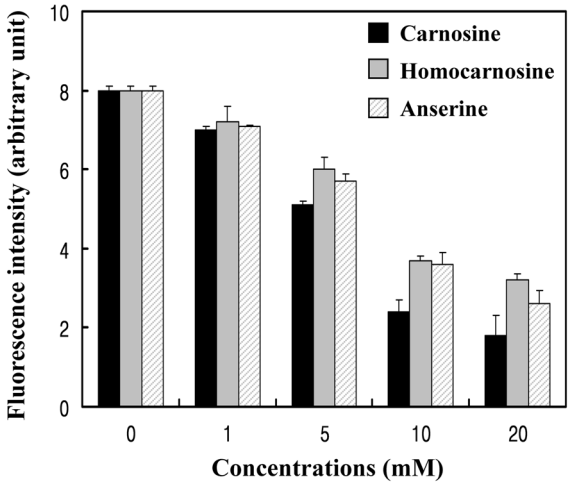

Figure 6. Effects of carnosine and related compounds on thioflavin T-bound NF-L aggregates. NF-L (0.15 mg/mL) was incubated with 1 mM acrolein in the presence of various concentrations of carnosine, homocarnosine and anserine.

in Figure 5, the formation of amyloid-like fibrils of NF-L was increased in the acrolein-concentration dependent manner. The protective effects of carnosine, homocarnosine and anseine on the formation of amyloid-like compounds were shown in Figure 6. Carnosine and related compounds may effectively prevent the formation of amyloid-like fibrils of NF-L by acrolein.

Previous studies have demonstrated that the ability of carnosine to quench the highly cytotoxic lipid peroxidation-derived 4-hydroxy-2-nonenal through the formation of two conjugated products, characterized by ESI-MS/MS analysis as a Michael adduct (involving the imidazolic nitrogen of histidine) and an immine adduct involving the β-alanine residue.[35] Recently, it was demonstrated that carnosine and homocarnosine detoxify the highly reactive aldehyde acrolein in a buffer system.[36] Human brain contains carnosine and its analogous, although with different localization, carnosine being specifically located in the olfactory bulb and in glial cells, while its analogous are widespread in whole brain tissue and in the cerebrospinal fluid.[4] More recently, it was reported that carnosine can suppress amlyloid-beta peptide toxicity[37] and carnosinase activity significantly decreased in AD and PD.[38] Thus carnosine and related compounds should be explored for therapeutic potential towards neurodegenerative disorders such as AD and PD.

In conclusion, the present results suggest that carnosine and related compounds can prevent the acrolein-mediated NF-L modification through the combination of scavenging of carbonyl compounds and anticrosslinking. Therefore, carnosine and related compounds may effectively suppress the pathogenesis of degenerative disorders which may be closely associated with the abnormal modification of neurofilaments.

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