Protective Effects of Carnosine and Anserine on Oxidative Modification of Neurofilament-L Induced by Catechol Neurotoxin, Tetrahydropapaveroline

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Received November 19, 2011, Accepted December 30, 2011

Tetrahydropapaveroline [THP; 6,7-dihydroxy-1-(3,4-di-hydroxybezyl)-1,2,3,4-tetra-hydroisoquinoline)], a metabolite of dopamine, was implicated in the development of Parkinsons disease (PD) and some side effects derived from its treatment with L-dopa.[1] Previous studies indicated that THP was toxic to dopaminergic neurons both *in vitro* and *in vivo* conditions, which suggested that THP-induced apoptosis was directly related to the potential oxidative stress caused by the oxidation of the catechol moiety of THP.[2] Recent report also revealed that THP autoxidation occurred with a continuous generation of reactive oxygen species (ROS).[3]

Neurofilaments (NFs) are the most abundant cytoskeletal element in axons.[4,5] The three mammalian neurofilament subunits, NF-L (~68 kDa), NF-M (~145 kDa), and NF-H (~200 kDa), were proposed to form heterodimers, consisting of NF-L in combination with either NF-M or NF-H.[6,7] NFs were implicated in the pathogenesis of several neurodegenerative disorders. Recent progresses in Parkinson's disease (PD) research indicated that PD could be caused by protein aggregations from aberrant protein folding or disturbance in protein degradation.[8] NF proteins were identified immuno-histochemically as the major protein components of Lewy body.[9] Abnormal accumulations of NF in neurons was associated with other neurodegenerative diseases, such as Alzheimer's disease and amyotrophic lateral sclerosis.[10,11] Above studies implied that NF-L could be accumulated in some tangle-like structures in the brains of Alzheimer's disease patients. However, the factors and mechanisms for the aggregated NF remained to be elucidated.

Carnosine (β-alanyl-L-histidine) is an antioxidant molecule, was found in the brain, muscle and other excitable tissues, exclusively in animal and humans. Carnosine could act as a copper chelator, and it was known to induce rejuvenating effects for participating in neuroprotective actions, such as preserving cell, suppressing cell senescence, and possessing anti-cross linking properties.[12,13] Several studies suggested that exogenously administrated carnosine had a neuroprotective effects against brain injury resulting from a variety of different causes.[14-16] Additionally, carnosine possessed a low toxicity, which were verified in mammalian systems. These properties suggested that carnosine could be explored, as a potential therapeutic agents for overcoming human neurodegenerative disease. Although THP was associated with the pathogenesis in neurodege-

rative diseases, the THP induced modification of NF-L and the effects of carnosine on THP-mediated NF-L modification had not yet been reported. In this study, we examined the THP induced modification of NF-L, then the protective effects of carnosine and anserine were further investigated.

To determine whether the modification of NF-L was induced by THP, the reaction mixtures containing NF-L and THP were subjected to SDS-PAGE analysis. When NF-L was incubated with various concentrations of THP for 6 h, protein aggregation was increased in a THP concentration-dependent manner (Fig. 1). NF proteins, specifically ex-
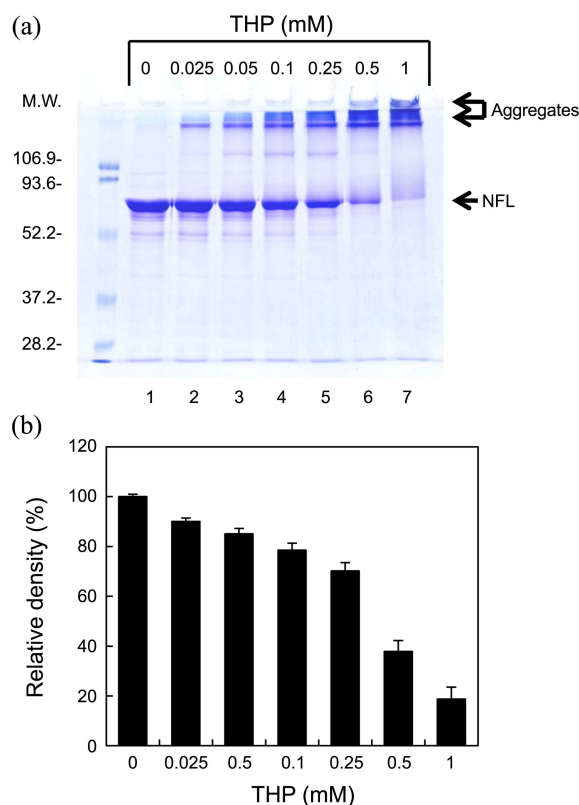

Figure 1. THP induced modification of NF-L. (a) NFL (0.4 mg/mL) was incubated with various concentrations of THP for 6 h. Lane 1, NF-L + 0 mM THP; lane 2, NF-L + 0.025 mM THP; lane 3, NF-L + 0.05 mM THP; lane 4, NF-L + 0.1 mM THP; lane 5, NF-L + 0.25 mM THP; lane 6, NF-L + 0.5 mM THP; lane 7, NF-L + 1 mM THP. (b) Relative staining intensity of SDS-PAGE gel was analyzed by densitometric scanning. The positions of molecular weight markers (kDa) were indicated on the left.

pressed in neuron and axons, could be implicated in a range of neurodegenerative disorders, including Alzheimer's disease, amyotrophic lateral sclerosis and Parkinson's disease.[10,11,17] The common feature in all these disorders would be the slow abnormal accumulation of NF aggregates in neuronal cells, accompanied by cell death.

The toxicity of THP may be augmented by its ROS-generating function in neurodegenerative disorder. Recent report has been revealed that THP could increase the production of ROS and induced oxidative PC cells death.[18] Since ·OH is the most powerful oxidizing species among several ROS, ·OH is able to oxidize most macromolecules, such as DNA, protein, lipid, and carbohydrate. Attack of ·OH on the sugar 2-deoxyribose could produce a huge variety of different products, some of which were mutagenic in bacterial systems. Some of the fragmentation products through the oxidation of biological molecules, could be detected by adding thiobarbituric acid (TBA) to the reaction mixture, forming of a pink (TBA)$_2$-MDA chromogen.[19] Above reaction could be used to detect ·OH productions, however it would not be clear whether or not some other ROS coould also degrade deoxyribose. To examine ·OH generation during the reaction of THP with NFL, we determined the formation of thiobarbituric acid reactive substance (TBARS). When 2-deoxyribose was incubated with various concentrations of THP, TBARS correlated directly with increasing THP (Fig. 2). These results suggested that ROS could participate in THP-mediated NFL modification with concentration dependent manner. Our data provided indirect evidence for the recent hypothesis of NFL

involvement in several neurological disorders.[20]

Carnosine and its related compounds were previously identified as efficient antioxidant molecules.[21,22] We found that carnosine and anserine effectively prevented NF-L aggregation (Fig. 3). Both compounds also inhibited the formation of carbonyl compounds (Fig. 4) and hydroxyl radicals (Fig. 5). Because carnosine and its related compounds were excellent free radical scavengers, the observed ability of those compounds in preventing the formation of protein carbonyls may be attributable to their free radical-scavenging activity. Additionally, carnosine was able to react non-enzymatically with carbonyl groups in proteins;
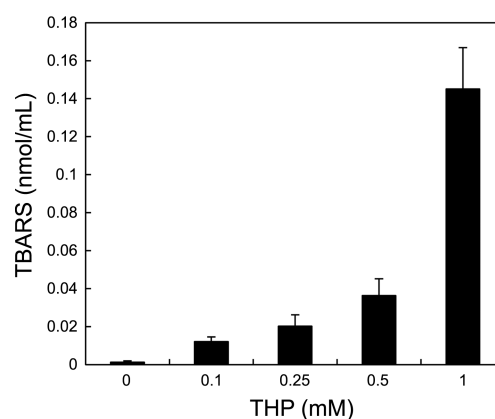

Figure 2. Detection of hydroxyl radicals. The reaction mixtures contained 10 mM 2-deoxy-D-ribose and various concentrations of THP (0, 0.1, 0.25, 0.5, 1 mM). Reaction mixtures were incubated at 37 °C for 6 h.
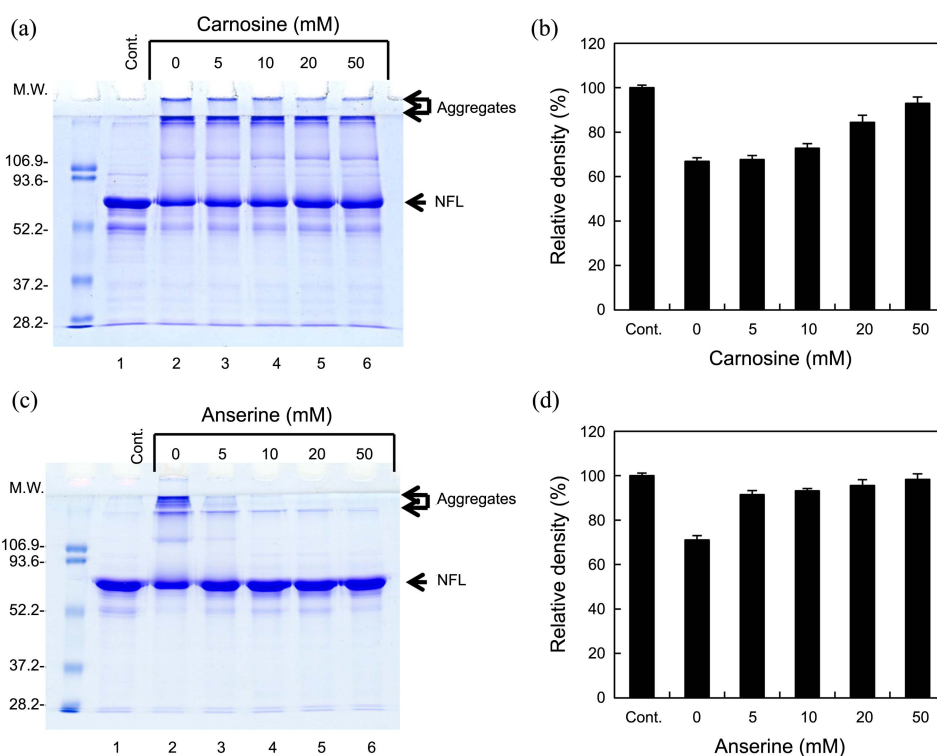

Figure 3. Effects of carnosine and anserine on THP-mediated NF-L modification. NF-L (0.4 mg/mL) was incubated with 0.25 mM THP in 10 mM potassuim phosphate buffer (pH 7.4) at 37 °C and was incubated for 6 h in various concentrations of carnosine and anserine. Reaction mixtures were analyzed by SDS-PAGE (a and c). Relative staining intensity of SDS-PAGE gel was analyzed by densitometric scanning (b and d). The positions of molecular weight markers (kDa) were indicated on the left.
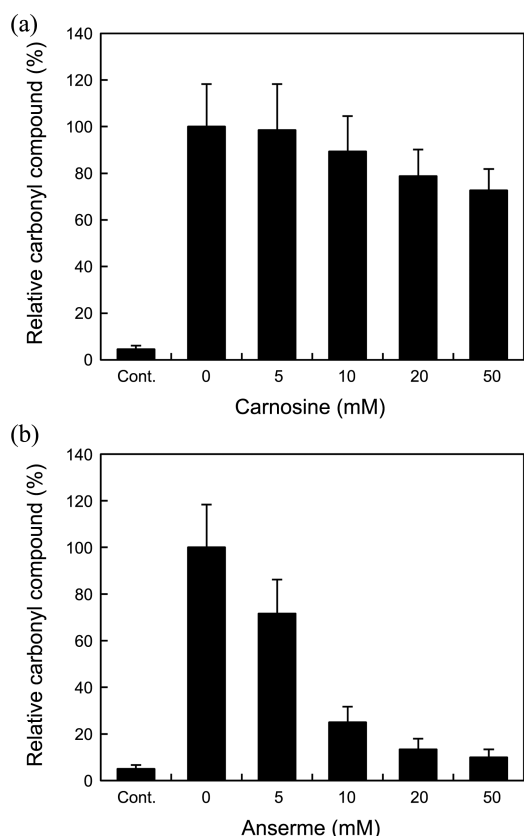
Figure 4. Effects of carnosine and anserine on the generation of carbonyl compounds. The reaction mixtures contained 1 mM of THP in 10 mM phosphate buffer at pH 7.4 and were incubated for 6 h in various concentrations of carnosine (a) and anserine (b).
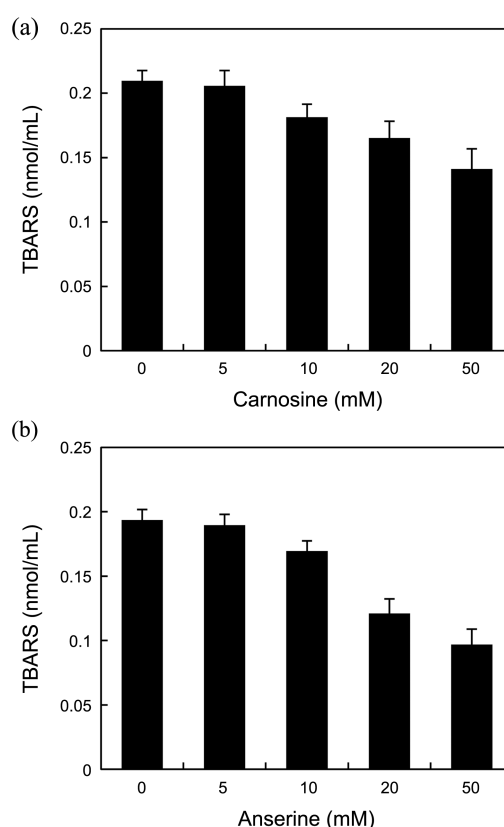

Figure 5. Effects of carnosine and anserine on the generation of hydroxyl radicals. The reaction mixtures contained 10 mM 2-deoxy-D-ribose and 1 mM of THP in 10 mM phosphate buffer at pH 7.4 and were incubated for 6 h in various concentrations of carnosine (a) and anserine (b).

this process was referred to as "carnosylation".[23] Prevention and removal of protein carbonyls may play a pivotal role in the protection of NF-L against oxidative stress. Accumulation of carbonyl compounds was identified as biological marker for oxidative damage in proteins,[24] and ROS was suggested to play a role in the formation of carbonyl compounds.[25] The results of this study demonstrated that carnosine and its related compounds may effectively protect NF-L against oxidative damage by neurotoxins, such as THP.

In conclusion, the findings of the current study demonstrated that THP induced the oxidative modification of NF-L through ROS generation. THP-mediated NF-L modification could be associated with the pathogenesis of PD, as well as to for other neurological disorders. Our results also indicated that carnosine and its constituents may function as effective scavengers of ROS, as well as, inhibitors of NF-L modification. Recently, *in vitro* and *in vivo* studies have demonstrated that carnosine could exert neuroprotective effects through variety of mechanisms.[26,27] Therefore, our results suggested that carnosine could be used as therapeutic agents, inhibiting THP-mediated neurodegenerative disorders.

Experimental Section

Materials. Tetrahydropapaveroline [THP; 6,7-dihydroxy-1-(3,4-dihydroxybezyl)-1,2,3,4-tetra-hydroisoquinoline)],

2,4-dinitrophenyl hydrazine (DNPH), carnosine and anserine 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.

Expression and Purification of Neurofilament-L. A full-length cDNA clone of mouse NF-L in a pET-3d vector was a generous gift from Dr. Beckman (University of Alabama). To express the recombinant protein, the vector was transformed into *E. coli* (BL21). Protein expression was performed as previously described.[28] Bacteria were grown in Luria broth supplemented with 1 mM isopropyl β-D-thiogalactopyranoside in the 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 × g for 10 min at 4 °C), and resuspended in standard buffer (50 mM MES, 170 mM NaCl, 1 mM DDT, pH 6.25). Cells were lysed with a French press at a pressure of 20,000 pounds per square inch 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 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 NF-L eluted between 300 and 360 mM phosphate. These fractions were pooled and were used directly or stored at −70 °C for later experiments. Protein concentration was determined by the BCA method.[29]

Oxidation of Protein. Oxidative modification of NF-L (0.4 mg/mL) was carried out by incubating of the protein with THP in 10 mM potassium phosphate buffer (pH 7.5) at 37 °C. After incubation of the reaction mixtures, the mixtures were then placed into Vivaspin ultrafiltration spin column and centrifuged at 13,000 rpm for 1 h. The column was washed with Chelex 100 treated water and centrifuged for 1 h at same speed. These steps were repeated four times. The filtrate was dried with freeze drier and again dissolved with phosphate buffer.

Analysis of Modified Protein. After treatment with various concentrations of THP for 6 h, samples from the reaction mixtures were diluted with a concentrated sample buffer (0.25 mM Tris, 40% glycerol, 0.01% bromophenol blue). Each sample aliquot was subjected to SDS-PAGE as described by Laemmli,[30] using a 12% acrylamide slab gel. Gels were stained with 0.15% Coomassie brilliant blue R-250.

Detection of Protein Carbonyl Compound. The protein carbonyl content were determined using spectrophotometric assays, as described elsewhere.[31] Both native and oxidized proteins were incubated with 10 mM 2,4-DNPH in 2.5 M HCl for 1 h at room temperature. After incubation, 20% TCA were added to the sample and the tubes were left in an ice bucket for 10 min. Afterwards, tubes were centrifuged for 5 min with a tabletop centrifuge and the supernatant were discarded to collect the protein precipitates. Another wash was performed using 10% TCA, and the protein pellets were mechanically broken with a pipette tip. Finally, the pellets were washed 3 times with ethanol-ethyl acetate (1:1) (v/v) to remove any free DNPH. The final precipitates were dissolved in 2 mL of a 6 M guanidine hydrochloride solution and left for 10 min at 37 °C with general vortex mixing. The carbonyl contents were calculated from the absorbance at 370 nm using an extinction coefficient e of $22{,}000\ \mathrm{M^{-1}\ cm^{-1}}$.

Measurement of Hydroxyl Radicals. Detection of hydroxyl radicals was performed by measuring thiobarbituric acid reactive substances (TBARS) from a modification of the method previously described.[32] An assay mixture would contained 10 mM potassium phosphate buffer (pH 7.4), 10 mM 2-deoxy-D-ribose, and THP in a total volume of 100 μL. Reaction mixtures were incubated at 37 °C for 3 h. The degradation of 2-deoxy-D-ribose was measured by adding of 2.8 % trichloroacetic acid (200 μL), PBS (200 μL), and 1% thiobarbituric acid (200 μL), followed by heating at 100 °C for 15 min. After cooling to room temperature, the samples were centrifuged at 15,000 rpm for 10 min. Results were read by a UV/vis spectrophotometer (Shimadzu, UV-1601) at 532 nm.

Statistical Analysis. Values were expressed as the means ± S.D from three to five separate experiments. The statistical differences between the means were determined by the Student *t*-test.

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