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Short communication

Protective Effects of Histidine Dipeptides on the Modification of Neurofilament-L by the Cytochrome *c*/Hydrogen Peroxide System

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Neurofilament-L (NF-L) is a major element of the neuronal cytoskeleton and is essential for neuronal survival. Moreover, abnormalities in NF-L result in neurodegenerative disorders. Carnosine and the related endogeneous histidine dipeptides prevent protein modifications such as oxidation and glycation. In the present study, we investigated whether histidine dipeptides, carnosine, homocarnosine, or anserine protect NF-L against oxidative modification during reaction between cytochrome *c* and H_2O_2. Carnosine, homocarnosine and anserine all prevented cytochrome *c*/H_2O_2-mediated NF-L aggregation. In addition, these compounds also effectively inhibited the formation of dityrosine, and this inhibition was found to be associated with the reduced formations of oxidatively modified proteins. Our results suggest that carnosine and histidine dipeptides have antioxidant effects on brain proteins under pathophysiological conditions leading to degenerative damage, such as, those caused by neurodegenerative disorders.

Keywords: Carnosine, Cytochrome *c*, Modification, Neurofilament-L

Introduction

Previous studies have suggested that abnormal protein aggregation is associated with the pathogenesis of neurodegenerative disorders (Lansbury, 1999). In Parkinson's disease (PD), a neurodegenerative disorder associated with dopaminergic nerve cell loss and the presence of neuronal inclusion bodies and dystrophic neurites in the substantia nigra and various other brain regions (Forno, 1996). Lewy bodies (LBs), which are cytoplasmic inclusions, are characteristically present and show greatest frequency in the neurons of the substantia nigra and locus ceruleus of PD patients (Forno, 1986). Ultrastructurally, LBs are visualized as being composed primarily of a filamentous mesh that surrounds a compact core. Moreover, all three neurofilament (NF) subunit proteins, i.e., heavy (NF-H), medium (NF-M), and light (NF-L), have been identified immunohistochemically as major components of LB filaments (Pollanen *et al*., 1993; Trojanoski *et al*., 1993).

Cytochrome *c* is an electron transport protein which catalyses peroxidase-like reactions *in vitro* (Radi *et al*., 1991a). Moreover, defective mitochondrial function has been found in brain, muscle and peripheral tissues, such as, platelets and fibroblasts in PD (Parker and Swerdlow, 1998). And, oxidative stress has been identified to be a factor involved in the release of cytochrome *c* from mitochondria to cytosol (Reed, 1997; Stridh *et al*., 1998; Ma *et al*., 1999), which is component of the trigger initiating the cascade of events that lead to apoptosis. Not surprisingly, increased levels of apoptosis have been reported to be associated with neurodegenerative diseases (Gorman *et al*., 1996; Schapira, 1999). Therefore, we hypothesized that oxidative damage to cytochrome *c* may increase during neurodegenerative diseases like PD.

Carnosine (β-alanyl-L-histidine), a natural occurring dipeptide, was first discovered by Gulewitsch and Amiradzibi (1900) in meat extract in 1900. Carnosine accumulates in the excitable tissues (brain and skeletal muscle) of vertebrates in large amounts and may reach more 10 mM per Kg (wet weight) (Abe, 1995). Moreover, several biochemical studies have concluded that carnosine possesses antioxidant and free radical-scavenging functions, which may partly explain its apparent homeostatic function (Auroma *et al*., 1989; Hartman *et al*., 1990). In addition, some related compounds, i.e., anserine (β-alanyl-3-methyl-L-histidine) and homocarnosine (γ-amino butyryl-L-histidine), have been reported to be present at millimolar concentrations in several mammalian tissues, including brain and skeletal muscle, although they show interesting differences in tissue distribution (Crush, 1970; O'Dowd *et al*., 1988). These derivatives have also shown antioxidant, proton buffering, or heavy metal chelating abilities in simple chemical models (Bolyrev *et al*., 1995; Boldyrev

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and Abe, 1999), but their biological activities have not been clarified. Although many studies have been conducted on the protective effects of carnosine and carnosine-related compounds on oxidative proteins damage, the inhibitory actions of these compounds on cytochrome *c*-mediated NF-L modifications are unknown. In the present study, we examined the protective effects of carnosine, homocarnosine, or anserine on cytochrome *c*/H_2O_2-mediated NF-L aggregation.

Materials and Methods

Materials. Cytochrome *c*, carnosine, homocarnosine, anserine, ethylene glycol-bis(2-amino ethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), dithiothreitol (DTT), sodium dodecyl sulfate (SDS), and monoclonal anti-neurofilament 68 antibody (mouse) were purchased from Sigma Chemical Co. Chelex 100 resin (sodium form) was obtained from Bio-Rad.

Preparation of neurofilament-L. The protein expression of NF-L and its purification were performed as previously described (Kim and Kang, 2003). Full-length cDNA clones of mouse NF-L in pET-3d vector were transfected into *E. coli* (BL21), and transfected bacteria at an OD 600 nm of 0.8 were grown in Luria broth supplemented with 1mM isopropyl β-D-thiogalactopyranoside. Incubation was performed at 37°C for 3 h. Bacteria were then 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), disrupted using a French press at 20,000 p.s.i., and centrifuged at 8,000 × *g* for 15 min at 4°C. 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 standard buffer before being dissolved in urea buffer (2 mM Na-phosphate, pH 7.5, 6 M urea, 1 mM EGTA and 1 mM DTT). Samples were loaded onto a DEAE-Sepharose column and eluted with a linear 25-500 mM phosphate gradient in urea buffer; 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 using the BCA method (Smith *et al*., 1985).

Analysis of NF-L modification. NF-L was oxidatively modified by incubating NF-L (0.25 mg/ml) with 10 mM cytochrome *c* and 500 mM H_2O_2 in 10 mM phosphate buffer (pH 7.4) at 37°C. Reactions were stopped by freezing at −80°C, and then treated with a 4× concentrated sample buffer (0.25 mM Tris-HCl, 8% SDS, 40% glycerol, 20% b-mercaptoethanol, 0.01% bromophenol blue) and heated in boiling water for 10 min. An aliquot of each sample was then subjected to SDS-polyacrylamide gel electrophoresis (PAGE), as described by Laemmli (1970), using 12% acrylamide slab gel. For immunoblotting, proteins on polyacrylamide gels were electrophoretically transferred to nitrocellulose membranes which were then 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) (Eum *et al*., 2005). Membranes were incubated for 1 h at room temperature with anti-NF antibody (1 : 400) in TTBS, washed with TBS, and re-incubated with peroxidase labeled secondary antibody. The protein bands were visualized using an enhanced chemiluminescence kit (ECL; PerkinElmer).

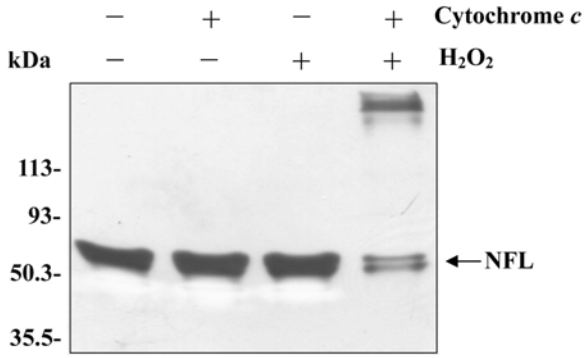

Fig. 1. Aggregation of NF-L by the cytochrome *c*/H_2O_2 system was analyzed by immunoblotting. NF-L (0.25 mg/ml) was incubated at 37°C for 2 h: Lane 1, NF-L control; lane 2, NF-L + 10 μM cytochrome *c*; lane 3, NF-L + 0.5 mM H_2O_2; lane 4, NF-L + 10 μM cytochrome *c* + 0.5 mM H_2O_2. Reactions were stopped by freezing at −80°C.

Detection of O,O'-dityrosine. O,O'-dityrosine was detected using NF-L (0.25 mg/ml), 10 μM cytochrome *c* and 500 μM H_2O_2 in a total volume of 300 μl. Briefly, samples were diluted with 2.7 ml of Chelex 100-treated water and transferred to a cuvette (3 ml). Sample fluorescence was monitored at 410 nm (emission) and at 325 nm (excitation) using a fluorescence 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 and Discussion

Figure 1 shows an original intact NF-L band after incubation with 10 μM cytochrome *c* or 500 μM H_2O_2. However, when NF-L was incubated in a mixture of cytochrome *c* and H_2O_2 protein aggregation occurred. Thus, indicating that both cytochrome *c* and H_2O_2 are required for the aggregation of NF-L. It was postulated that hydrogen peroxide, like peroxidases, oxidizes cytochrome *c* to an oxoferryl derivative. The oxoferryl cytochrome *c* then catalyzes the oxidation of biomolecules (Radi *et al*., 1991a; Radi *et al*., 1993b; Kim and Kang, 2006). Therefore, we reasoned that an oxoferryl derivative may be involved in cytochrome *c*-mediated NF-L aggregation. Moreover, it has been suggested that mitochondrial dysfunction may be involved in the pathogenesis of neurodegenerative disorders. (Schapira, 1994) The present study suggests that oxidative damage of NF-L by the cytochrome *c*/H_2O_2 system might occur in diseases like Parkinson's disease, associated with mitochondrial dysfunction.

Carnosine and its related compounds have been proposed to act as antioxidants, free radical scavengers, physiological buffering agents, neurotransmitters, radioprotectants, metal

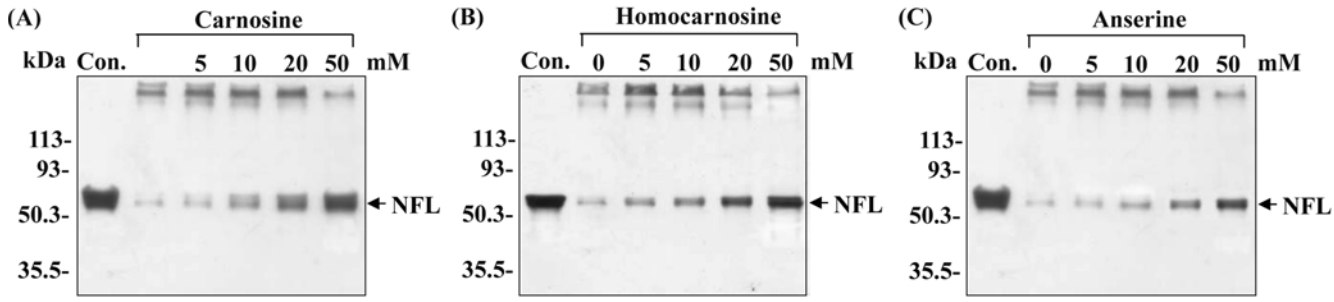

Fig. 2. Effects of carnosine, homocarnosine, or anserine on the aggregation of NF-L by the cytochrome *c*/H_2O_2 system. NF-L was incubated with 10 μM cytochrome *c* and 0.5 mM H_2O_2 in various concentrations of carnosine (A), homocarnosine (B), or anserine (C) at 37°C for 2 h. Lane 1, cytochrome *c* control; lane 2, incubated with H_2O_2; lane 3, 5 mM effectors; lane 4, 10 mM effectors; lane 5, 20 mM effectors; lane 6, 50 mM effectors. Protein oligomerization was analyzed by immunoblotting.

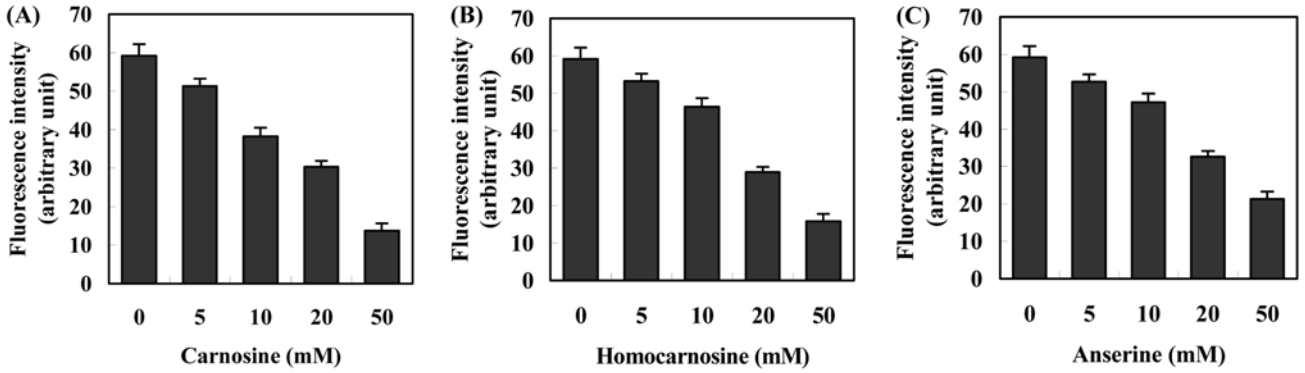

Fig. 3. Effects of carnosine, homocarnosine, or anserine on the formation of dityrosine in cytochrome *c*/H_2O_2-induced NF-L aggregates. 10 μM cytochrome *c* was incubated with 0.5 mM H_2O_2 in the presence of various concentrations of carnosine, homocarnosine, or anserine at 37°C for 2 h. Reaction mixtures were analyzed by fluorescent spectrometry. Data represent means ± SD (n = 3-5).

chelators, and wound healing agents (Brown, 1981; Boldyrev *et al*., 1988; Auroma *et al*., 1989). In the present study, it was found that carnosine, homocarnosine, and anserine all significantly inhibited the NF-L aggregation induced by the cytochrome *c*/H_2O_2 system (Fig. 2). In addition, it has been reported that imidazole-containing peptides, which are also carnosine and related compounds, may react with di- or monoaldehydes (powerful cross-linking agents) released during the oxidative breakdown of unsaturated lipids (Aldini *et al*., 2002). Our data suggest that the imidazolium group of carnosine might inhibit the formation of the oxoferryl derivative. Along with carnosine, homocarnosine and *N*-acetyl-histidine are accumulated in normal brain, whereas anserine and ophidine (N^1- and N^3-methylated carnosine derivatives, respectively) specifically accumulate in the skeletal muscles of birds, some fishes, and marine mammals (Abe, 1995). Therefore, in brain, carnosine and homocarnosine may play a critical role in the protection of proteins against oxidative damage. We also investigated whether carnosine and its related compounds could inhibit dityrosine formation. When NF-L was incubated with cytochrome *c* and H_2O_2 in the presence of carnosine, homocarnosine or anserine, they were found to effectively inhibit dityrosine formation (Fig. 3). Dityrosine is a biomarker of protein oxidation (Giulivi and Davies, 1993; Heinecke and Daehnke 3rd, 1993; Huggins *et al*., 1993) and is found in the amino acid hydrosylates of brain tissues affected by neurodegenerative disorders (Pennathur *et al*., 1999). Moreover, recent studies have suggested that the oxidative cross-linking of proteins by tyrosine residues occur via a peroxidase-like mechanism. The proposed mechanism for the reaction between H_2O_2 and ferricytochrome *c* owes a great deal to our knowledge of the reaction between H_2O_2 and the heme-containing peroxidase enzymes (Kato *et al*., 2001; Atwood *et al*., 2004). If two adjacent tyrosine radical-containing molecules interact, a dityrosine-linked dimer is formed. It has been reported that the exposure of α-synuclein (a neuronal presynaptic protein) to nitrating agents led to tyrosine oxidation to form o,o'-dityrosine, and crosslinking of α-synuclein to form stable high molecular mass α-synuclein aggregates (Olteanu and Pielak, 2004). NF-L is susceptible to oxidation because it is among the most abundant proteins in neuronal cells and because it contains a large molar fraction of tyrosine. Our data suggest that carnosine, homocarnosine, and anserine protect against NF-L aggregation caused by oxidative damage by cytochrome *c*/H_2O_2 by inhibiting dityrosine formation.

The data presented here are consistent with the inhibition of the cytochrome c/H_2O_2-mediated modification of NF-L by carnosine, homocarnosine, or anserine. Our findings indicate that these compounds should be further explored as potential therapeutic agents that counteract the oxidative stress associated with neurodegenerative disorders like Parkinson's disease.

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