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Formation of high-molecular-weight protein adducts by methyl docosahexaenoate peroxidation products

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Abstract

In the present study, the formation of modified proteins by methyl docosahexaenoate (DHA) peroxidation products in the presence of a metal-catalyzed oxidation system was investigated. Metal-catalyzed oxidation of mixtures containing bovine serum albumin (BSA) and DHA led to formation of two high molecular weight derivatives of BSA. One had a mass of 71.5 kDa as determined by two-dimensional electrophoresis, matrix assisted laser desorption and ionization mass spectrometer (MALDI MS) analysis. The other was estimated to be 93 kDa by SDS-PAGE electrophoresis. The exposure of BSA to DHA also led to the generation of carbonyl groups. Oxygen radical scavengers could inhibit these modifications induced by DHA peroxidation. Furthermore, there was little difference of the peptides mass fingerprinting between the two kinds of modified high-molecular-weight proteins. These results suggest that oxygen radicals formed during lipid peroxidation are involved in the formation of protein derivatives. Our study may be important in the understanding the specific role of docosahexaenoic acid in the formation of modified proteins during aging and its related diseases.

Keywords: DHA peroxidation; Protein modification; Two-dimensional electrophoresis; Western blot analysis; ESR analysis; MALDI MS

1. Introduction

Docosahexaenoic acid is the most polyunsaturated fatty acid (PUFA) commonly present in biological membranes. Large quantities of docosahexaenoic acid are found in the brain gray matter and synaptic membranes. In particular, docosahexaenoic acid accounts for close to 80 mol% of the total fatty acid components of the outer segment membranes of retina rod cells [1]. Docosahexaenoic acid levels in both the brain and the retina are crucial for proper nervous system and vision development [2,3]. However, the high content of docosahexaenoic acid indicates the potential for harm, since a high intake of docosahexaenoic acid may increase a biological system's susceptibility to lipid peroxidation [4,5].

Lipid peroxidation has been implicated in the pathogenesis of numerous diseases including atherosclerosis, diabetes, cancer, and aging [6]. It is well established that these end-products of lipid peroxidation, such as malondialdehyde (MDA), 4-hydroxynonenal (HNE), and 4-hydroxyhexenal (HHE), cause protein damage by means of reactions with lysine amino groups, cysteine sulfhydryl groups, and histidine imidazole groups [7]. Modifications of protein by aldehyde products of lipid peroxidation are also believed to contribute to neuronal death in Alzheimer's disease [8–10]. Recent studies have shown that HNE is involved in stress signaling pathways through activation of c-Jun N-terminal kinases [11] and inhibition the NF-κB/Rel system [12].

While all of these studies support the notion that PUFAs in tissue are important sources for the formation of endogenous protein adducts, the relative contributions of different PUFAs, especially DHA, in the formation of these protein adducts have not yet been examined. In the previous study, we reported that the ability of unsaturated fatty acid methyl esters to modify BSA in the presence of a metal-catalyzed oxidation system was

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strongly dependent on the degree of fatty acid unsaturation [13]. Here, we investigated further the formation of modified proteins by DHA peroxidation products in the presence of a metal-catalyzed oxidation system and found oxygen radicals formed during lipid peroxidation are involved in the formation of high-molecular-weight protein derivatives. The results shown here may be important in understanding the specific role of docosahexaenoic acid in the formation of degenerative proteins during aging and its related diseases.

2. Materials and methods

2.1. Materials

BSA was obtained from Roche, Switzerland. Cytochrome C, myoglobin, and 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide (DEPMPO) were obtained from Alexis, USA. DHA was obtained from Nu-Chek Prep., USA and purified by preparative thin-layer chromatography (TLC) just before use. Anti-acrolein (ACR) antibody, anti-crotonaldehyde (CRO) antibody, anti-MDA antibody, anti-HHE antibody, and anti-HNE antibody were obtained from NOF Corporation, Japan. Pure nitrocellulose membranes and horseradish peroxidase-linked goat anti-rabbit IgG immunoglobulin were obtained from Bio-Rad. Supersignal ULTRA chemiluminescent substrate was obtained from Pierce. 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide (DEPMPO) was obtained from Alexis, USA. Mannitol, histidine, sodium benzoate, KI were of analytical grade.

2.2. Reaction of protein with methyl docosahexaenoate

Protein (1 mg/ml) was incubated with DHA (1.0 mM) in 50 mM HEPES buffer (pH 7.4) containing 0.2% (w/v) of Tween 20 at 37 °C. Mannitol, histidine, sodium benzoate, or KI was added at final concentration of 0.5 M, 0.2 M, 0.5 M, or 0.1 M, respectively. Reactive oxygen species were generated by addition of $FeSO_4$ (1 μM) and ascorbic acid (AsA, 20 μM).

2.3. Preparation of aldehyde-modified BSA

200 μl of acrolein (~2.993 mmol) or crotonaldehyde (~2.414 mmol) was diluted with equal volume of ethanol, then incubated with 2 ml of BSA (1 mg/ml) in 50 mM HEPES buffer (pH 7.4) at 37 °C for 24 h. Malondialdehyde modified BSA was prepared as follows: 200 μl of tetramethoxypropane (~1.218 mmol) was taken and 1 ml of 6 N HCl was added to the tube and the tube was heated at 60 °C for 30 min. The pH was adjusted to 7.2 using 4 N NaOH, and the total volume adjusted to 1.4 ml using 50 mM HEPES buffer, then incubated with 1 ml of BSA (2 mg/ml) at 37 °C for 24 h [14].

2.4. Protein carbonyl formation

An aliquot of the protein samples was incubated for 1 h with an equal volume of 0.1% (w/v) 2, 4-dinitrophenylhydrazine (DNPH) in 2 N HCl. After precipitation with trichloroacetic acid, the pellet was washed three times with ethanol/ethyl acetate (1:1, v/v). The protein sample was then dissolved in 8 M guanidine hydrochloride, 13 mM EDTA, 133 mM Tris (pH 7.4), and the UV absorbance was measured at 370 nm [15].

2.5. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Laemmli [16]. The protein was stained with Coomassie Brilliant Blue.

2.6. Two-dimensional electrophoresis

Isoelectric focusing was performed on pH 3–10 nonlinear immobilized pH gradient (IPG) strips. The strips were rehydrated overnight in a solution consisting of 8 M urea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonic acid (CHAPS), 50 mM 1, 4-dithiothreitol (DTT), and 2% IPG buffer (pH 3–10) containing the protein sample. The running conditions on the gels were 1 h at 250 V, 1 h at 500 V, 1 h at 1000 V, 1 h at 2000 V, 4 h at 4000 V, 2 h at 500 V. Prior to the second dimension, strips were incubated in equilibration buffer (50 mM Tris–HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol) with 1% DTT for 15 min and then with 2.5% iodoacetamide in the same buffer without DTT for 15 min. The separation was performed in 10% SDS-PAGE gels using mini-PROTEAN III cell (Bio-Rad). The gels were Coomassie Brilliant Blue stained.

2.7. Western blot analysis

The proteins were blotted onto a nitrocellulose membrane and the membrane was treated with primary antibody overnight at 4 °C. Then the membrane was incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase. After incubation with supersignal ULTRA chemiluminescent substrate, the bands were visualized by exposure of the membrane to autoradiography film.

2.8. Electron spin resonance (ESR) measurement

The ESR samples were: 5 mM DHA; 0.2 mM or 5 mM BSA in PBS (50 mM, pH 7.4). Two minutes after introduction of spin trap DEPMPO to the sample, oxidative stress was initiated by adding $FeSO_4$ (100 μM or 4 mM) and the sample was immediately transferred into a capillary tube for ESR measurement. All ESR spectra were obtained with a Bruker EMX-8/2.7 spectrometer at room temperature. The ESR spectrometer settings were: modulation frequency 100 kHz; modulation amplitude 1.0 G; microwave power 20 mW; receiver gain 5×10^5. The ESR simulation program (Winsim v.1.0, 2002) is available upon request from Dr. J.J. Jiang freely (http://epr.niehs.nih.gov/pest.html). Fenton system was used as the control for hydroxy radical: 1 mM H_2O_2, 4 mM $FeSO_4$, 4 mM EDTA, 1% (v/v) for DEPMPO.

2.9. Molecular mass determination

The proteins were precipitated with acetone and then redissolved by 50% acetonitrile containing 5 mg/ml α-cyano-4-hydroxycinnamic acid and spotted directly. The MALDI MS was an ABI 4700 TOF-TOF Proteomics Analyzer instrument (Applied Biosystems, USA).

2.10. Peptide mass fingerprinting

The samples were separated on SDS-PAGE and the bands were visualized by staining with Coomassie Brilliant Blue. The bands were excised and digested by trypsin. Tryptic peptides were dissolved by 50% acetonitrile containing 5 mg/ml α-cyano-4-hydroxycinnamic acid and spotted directly.

3. Results

3.1. Protein modification

BSA was used to study the effects of polyunsaturated fatty acid esters on the oxidative modification of proteins because there is a large body of literature documenting its reactions with oxidants and because it contains no metal prosthetic groups [17]. In our study, proteins were detected by SDS-PAGE analysis after exposure to the Fe^{2+} and AsA mixed-function oxidation systems for various times, in both the presence and absence of DHA. As shown in Fig. 1A, there was no generation of a new protein band when BSA was exposed to the Fe^{2+} and AsA mixed-function oxidation system in the absence of DHA. However, the high-molecular-weight protein (Protein 3) band