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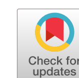
Non-enzymatic conversion of primary oxidation products of Docosahexaenoic acid into less toxic acid molecules



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ABSTRACT

Docosahexaenoic acid (DHA) is long chain omega-3 fatty acid with known health benefits and clinical significance. However, 4-hydroxy hexenal (HHE), an enzymatic oxidation product of DHA has recently been reported to have health-damaging effects. This conflict raises major concern on the long-term clinical use of these fatty acids. Even though the enzymatic and non-enzymatic conversion of HHE to nontoxic acid molecules is possible by the aldehyde detoxification systems, it has not yet studied. To address this, primary oxidation products of DHA in lipoxidase system were subjected to non-enzymatic conversion at physiological temperature over a period of 1 week. The reaction was monitored using HPLC, IR spectroscopy and biochemical assays (based on the loss of conjugated dienes, lipid peroxides aldehydes). Short term and long term cytotoxicity of the compounds generated at various time points were analyzed. IR and HPLC spectra revealed that the level of aldehydes in the primary oxidation products reduced over time, generating acids and acid derivatives within a week period. In short term and long term cytotoxicity analysis, initial decomposition products were found more toxic than the 1-week decomposition products. Further, when primary oxidation products were subjected to aldehyde dehydrogenase mediated oxidation, it generated products that are also less toxic. The study suggests the possible non-enzymatic conversion of primary oxidation products of DHA to less cytotoxic acid molecules. Exploration of the physiological roles of these acid molecules may explain the biological potential of omega-3 fatty acids.

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1. Introduction

Many epidemiological and experimental studies provide relatively strong evidence for the beneficial effects of omega-3 fatty acids in various human ailments [1–3]. Clinical uses of these fatty acids, however, are restricted to hypertriglyceridemia and some neurological ailments wherein relatively higher concentrations for a longer period of time are usually advised. Recently, increased lipid peroxidation products mediated toxic insults have been raised major concern over the long-term use of these fatty acids; however, the mechanistic basis for this has not been explained [4].

Docosahexaenoic acid (22:6, n-3) has been extensively studied, experimentally and clinically and conflicting reports exist regarding its pharmacological activity [5,6]. Omega-3 fatty acid derived compounds have been reported to have antioxidant potential mediated by the nuclear factor related erythroid factor 2 (Nrf2) [7]. In contrast, 4-hydroxy 2-hexenal (HHE), an oxidation product of omega-3 fatty acids, has been found to exert toxic signals other cell types including prostate, leukaemia, lung and liver cells [8] and is reported to be high in neuronal cells and brain tissue of neurodegenerative disease patients [9].

However, in the body, a strong detoxification system exists to decompose these primary oxidation products. Reduced glutathione, aldehyde dehydrogenases or reductase etc. are coordinately involved in the conversion or elimination of these molecules. It is also expected that these primary oxidation molecules can undergo an auto-decomposition process to form secondary products. Very little information is known about these secondary molecules. Identification of such an alternate pathway and understanding the pharmacological efficacy of the conversion products may lead to the development of newer drug candidate. With this view in the present study, we aimed to investigate the secondary molecules formed during the conversion of oxidized DHA at physiological temperature and the cytotoxicity of these molecules in normal as well as malignant cells.

Abbreviations: DHA, Docosahexaenoic acid; MDA, Malondialdehyde; DLA, Dalton's Lymphoma Ascites; EAC, Ehrlich's Ascites Carcinoma.

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2. Materials and Methods

2.1. Chemicals

Docosahexaenoic acid, Soya bean lipoxidase type V and MTT used in this study were purchased from Sigma Aldrich (St. Louis, MO, USA). TLC plates (Silica gel, 60G, F_{254}), acetonitrile, trypan blue, methanol and water used for the chromatographic procedures were of HPLC grade (Merck, India). All other chemicals used were of analytical quality. All the chemicals used in the study were free from exogenous antioxidant butylated hydroxytoluene (BHT).

2.2. Cell Lines

Dalton's Lymphoma Ascites (DLA) and Ehrlich's Ascites Carcinoma (EAC) cells maintained in the peritoneal cavity of Swiss albino mice at Amala Cancer Research Centre animal house facility were used. Splenocytes were isolated from male Sprague Dawley rat. Human monocytic leukaemia cells (THP-1) and African green monkey kidney cells (Vero) were purchased from NCCS, Pune. Vero cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum and THP-1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. These cells were maintained at 37 °C with 5% CO_2 and humidity.

2.3. Enzymatic Oxidation of DHA

The reaction system contained 100 μM DHA and 100 units/mL of Soya bean lipoxidase type V enzyme (LPX) in phosphate buffered saline (pH 7.4). The conjugated dienes ($\lambda_{max} = 234$ nm) formed in the reaction were monitored using a UV–Vis Spectrophotometer (T80+ UV/VIS Spectrophotometer, PG Instruments Ltd.) for 1 h. The resultant reaction products were extracted (1:1) using chloroform: methanol (2: 1 v/v). The solvent-free residue was dissolved in ethanol and diluted with PBS (pH 7.4) and further incubated over a 1 week time period at 37 °C. Samples were withdrawn at 0, 1, 24, 48, 72 h, and 1 week for analytical purposes.

2.4. Aldehyde Dehydrogenase Mediated Conversion

Aldehyde dehydrogenase mediation was carried out according to standard protocols [10]. Briefly, aldehyde dehydrogenase (100 U/mL) was added to mixture containing 193 μL of deionized water, 100 μL of 200 mM glycine-NaOH buffer, pH 9.5, 0.4% v/v Triton X-100, 40 μL of 100 mM pyrazole, 20 μL of 10 mg/mL bovine serum albumin (fatty acid free), 24 μL of 25 mM NAD. Each sample was incubated at 37 °C and the reaction initiated by adding 3 μL of 160 μM DHA oxidation products. ALDH activity was then be monitored by measuring the increase in absorbance at 340 nm per minute, the decomposition products after 1 h was extracted using the method described by Bligh and Dyer [11].

2.5. TLC Analysis of the Decomposition Products

The oxidized and decomposed products of EPA and DHA at various time points were separated on thin layer chromatography (TLC) using the solvent system, hexane: diethyl ether: acetic acid (80: 20: 1.5, v/v/v) [12].

2.6. Extraction of Non-enzymatic Conversion Product and HPLC Analysis

The secondary conversion products collected at various time points (0, 24, 48, 72 h and 1 week) were extracted as described above. Equal volumes of samples and chloroform: methanol (2: 1) was mixed and the organic layer was collected after centrifugation (10,000 rpm for 10 min). The procedure was repeated for 3 times to ensure complete extraction. The organic layer collected was pooled; concentrated and the solvent-free residue was dissolved in methanol (HPLC grade). This was subjected to RP-HPLC analysis using C18 column (100 × 4.6 mm) in a Shimadzu SCL-10A VP instrument set at 234 nm for 10 min. The mobile phase used was acetonitrile: methanol (50: 50 v/v) at a flow rate of 1 mL/min.

2.7. IR Spectrum Analysis

The samples dissolved in methanol (HPLC grade) were mixed with potassium bromide crystals and methanol content was completely dried. The IR spectrum was measured using FTIR spectrophotometer (Shimadzu, 8400S) between wavelength 3500–750 nm^{-1}. The percentage transmission against the wavelength was plotted.

The total area under the curve (AUC) of each of the peaks was calculated from the FTIR spectra plotted by wave number against its intensity, using the FTIR system software and used for the interpretation of the data.

2.8. Short-term Cytotoxicity Analysis

Short term cytotoxicity was determined by Trypan blue dye exclusion method [13]. Approximately 1×10^7 DLA, EAC or spleen cells were suspended in 1 mL PBS; pH 7.4. From this stock, 1×10^6 cells (100 μL) was added to different test tubes containing 800 μL PBS. Primary oxidized and secondary conversion products of DHA at different concentrations (10–100 μg/mL) were added to each test tube. Incubation was carried out for 3 h at 37 °C. At the end of the incubation, 100 μL trypan blue was added to each tube and further incubated for 3 min. The cell suspension (10 μL) was loaded on to a haemocytometer and observed under microscope. Live cells (non-stained cells) and dead cells (blue stained cells) were separately counted and percentage cell death was determined.

2.9. Long-term Cytotoxicity Assay Using MTT

The long-term cytotoxicity of the primary oxidized and its secondary products was determined using MTT assay (3- (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide). Approximately, 1×10^6 cells (THP-1 or Vero) were seeded on to the flat bottom of the 12 well plate containing 1 mL appropriate medium and at 80% confluency, cells were incubated with various concentrations (10–100 μg/mL) of primary oxidation and secondary conversion products of DHA for 48 h. After incubation, treatment medium was removed and fresh medium containing 0.5% MTT was added to each well and incubated for another 4 h. The formazan crystals formed were dissolved in the solubilizing solution (10% Triton X-100, 0.1 N HCl in 125 mL Isopropanol) and the absorbance of the resulting solution was measured at 570 nm in a UV/Vis Spectrophotometer (T 80^+, PG Instruments). The optical density of untreated control well was considered as 100% survival and percentage death of cells was calculated from the variation of OD in the treated wells.

3. Results

3.1. UV/Vis Analysis of Oxidation and Decomposition Product of DHA

The spectrophotometric analysis of the oxidation of DHA using lipoxidase V enzyme showed a distinct increase in the absorbance at 234 nm, which is an indication of the formation of conjugated dienes over a period of 1 h (Fig. 1i). Quantitative measurements of the oxidized products within this time period indicated that 90% fatty acids had undergone oxidation. Upon incubation at 37 °C, the primary oxidized products were found to undergo non-enzymatic conversion to generate secondary products as evidenced by the progressive reduction in the 234 nm peak (Fig.1ii).

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