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DOCOSAHEXAENOIC ACID ETHYL ESTER AND ITS ULTRAVIOLET DEGRADATION PRODUCTS SHOWED DNA-BREAKING ACTIVITY *IN VITRO* AND CYTOTOXIC EFFECTS ON THE HSC-4 CELL LINE

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INDEXING WORDS

docosahexaenoic acid; DNA-breaking activity; cytotoxicity

SYNOPSIS

The DNA-breaking activity and cytotoxicity of docosahexaenoic acid ethyl ester (DHAE) and its degradation products (dDHAE) induced by ultraviolet (UV) irradiation, were demonstrated in this study. The major component of dDHAE was identified as 4-hydroxyvaleric acid ethyl ester. DHAE and dDHAE solutions at a concentration of 100 mg/ml induced single-strand breaks of plasmid DNA after an incubation at 37°C and pH 7.6 for 15 hours. DHAE transformed a plasmid supercoiled DNA (Form I) into an open circular relaxed form (Form II), and dDHAE completely destroyed DNA molecules. HSC-4 cells, which were derived from human prickle cell carcinoma, were cultured with various concentrations (10, 30 and 50 μg/ml) of DHAE and dDHAE. Both inhibited the proliferation of HSC-4 cells. The potency of their cytotoxicity was dependent on their concentration. It is noteworthy that the lethal effects of DHAE and dDHAE on HSC-4 cells were quite similar, although the DNA-breaking activity of dDHAE was much greater than that of DHAE. These results suggested that DHAE becomes more cytotoxic after undergoing oxidization and metabolism in the cell.

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INTRODUCTION

Polyunsaturated fatty acids (PUFAs) in the cell membrane are the major site of peroxidative damage.[1,2] Intracellular accumulation of lipid peroxides derived from the autoxidation of the membrane PUFA reduces the deformability of erythrocytes, contributing to the homorheological disturbances observed in acute cerebral ischemia.[3] An increased proportion of docosahexaenoic acid (DHA) and a high lipid peroxidation capacity in erythrocytes of stroke patients have been reported.[4]

The harmful effects of peroxidation of the membrane PUFAs are not limited to the reduction of the deformability of cells. PUFAs are readily oxidized to hydroperoxides, which are further broken down into a variety of secondary oxidation products. Both the hydroperoxides and secondary products, including polymers, aldehydes, hydroperoxyalkenals and hydroperoxyepoxides, are toxic in biological systems.[5]

In this paper, firstly, we identified the major component of the degradation product (dDHAE) of DHA ethyl ester (DHAE), which has never been reported as a secondary product of DHA/DHAE. Secondly, we demonstrated that DHAE and dDHAE had DNA-breaking activity *in vitro* and cytotoxic action on a cell line derived from human prickle cell carcinoma, HSC-4. The lethal effects of DHAE and its degradation products on the HSC-4 cell line were quite similar, although the DNA-breaking activity of the degradation products of DHAE was much greater than that of DHAE itself.

MATERIALS AND METHODS

Materials

A prickle cell carcinoma cell line, HSC-4, was provided by Dr. Masato Ueda, Department of Dermatology, Kobe University School of Medicine. DHAE was obtained from Sigma Chemical Company (St. Louis, MO, USA). Potassium hydroxide, hydrocloric acid, gamma-valerolactone, N, O-Bis(trimethylsilyl)-trifluoroacetamide, chloroform, ethanol and tris(hydroxymethyl)aminomethane hydrochloride were from Nakarai Tesque, Inc. (Kyoto, Japan). Eagle-MEM with glutamine was from Nikken Bio Medical Laboratory (Kyoto, Japan). Fetal calf serum was from Hyclone Laboratories, Inc. (Logan, UT, USA). Plasmid pGEM®-3Zf(+) DNA solution (200 μg/ml) was from Promega (Madison, WI, USA).

Degradation of DHAE by UV irradiation

UV irradiation was applied using a germicidal 10 W-UV lamp (200 to 280 nm). DHAE dissolved in chloroform was exposed to the UV lamp at a distance of 8 cm for 60 min at room temperature. The degradation product of DHAE was blow-dried with nitrogen. DHAE and its degradation product after UV irradiation (dDHAE) were redissolved in ethanol and ready for analysis for identification of the compound, DNA-breaking test and addition to the medium for cell culture.

Synthesis of authentic compounds

4-Hydroxyvaleric acid ethyl ester was prepared by hydrolysis and esterification of gamma-valerolactone. Gamma-valerolactone (10mg) was hydrolysed in 5ml 0.1N KOH-methanol solution at 100°C for 3 hours, and then neutralized with 3N hydrochloric acid. From the reaction mixture, 4-hydroxyvaleric acid was extracted with chloroform. 4-Hydroxyvaleric acid was esterified in hydrogen chloride-ethanol at 100°C for one hour.

Gas chromatography-mass spectrometry analysis

Degradation products of DHAE and dDHAE were identified by means of gas chromatography-mass spectrometry (GC-MS). dDHAE and the authentic compounds was incubated with N, O-Bis (trimethylsilyl)-trifluoroacetamide at 80°C for 30 min. Trimethylsilyl (TMS) derivatives of dDHAE were applied to the GC-MS apparatus. Analysis was performed with a JEOL Model JMS DX-300 GC-MS (Nihon-denshi, Tokyo, Japan) with an on-line JEOL Model JMA 5000 mass data analysis system (Nihon-denshi). The compounds were separated on a 30 m x 0.53 mmID fused silica megabore column DB-1 (J. & W. Scientific, Inc., Folsom, CA, USA), with helium as the carrier gas (25 ml/min). The column temperature was programmed from 100°C to 285°C (8°C/min). Electron impact mass spectra were obtained at an ion source temperature of 250°C, an ionization potential of 70 eV, an ionization current of 300 μA and an acceleration voltage of 3.0 kV.

DNA-breaking activity

DNA-breaking activity was measured by the method of Hiramatsu et al[6] with some modifications. DHAE (or dDHAE) was diluted into ethanol to concentrations of 0.2, 2, 10 and 200 mg/ml. To a mixture of 1 μl of 1 M Tris (pH 7.6) and 3 μl of water, 1 μl of the plasmid DNA (200 μg/ml) and 5 μl of DHAE (or dDHAE) were added in that order. The mixture was incubated at 37°C for 3, 9 and 15 hours. Prior to electrophoresis, 3 μl of loading buffer (0.25% bromophenol blue and 50% glycerol) was added to each fragmented DNA sample in a 1:1 (v/v) ratio. DNA electrophoresis was done in 1% agarose gel soaked in TBE buffer for 45 min at 100 V. DNA was visualized with ethidium bromide.

Cell culture and cell viability study

HSC-4 cells were cultured in MEM with 10% fetal calf serum at 37°C in a 5% CO_2 humidified incubator. HSC-4 cells were seeded at 0.3 x 10^5 cells/ml per tissue culture plate. One day after seeding, the medium was removed and fresh medium with and without DHAE or dDHAE was added. DHAE and dDHAE were dissolved in ethanol and the final concentration of ethanol was 0.01% of the new medium. Cells incubated for 24 hours were examined morphologically under phase contrast microscopy. Adherent cells were counted as viable cells with hemocytometer after one, two and three day-incubation with these compounds. All experiments were performed in triplicate and repeated at least twice.

RESULTS

The total ion chromatograms (TIC) of DHAE and dDHAE obtained by GC-MS analysis are shown in Fig. 1A. The TIC showed that intact DHAE was not present after UV-irradiation for 1 hour, and a compound with a short retention time appeared as an intense peak. The mass spectra of DHAE and the major component of dDHAE-trimethylsilyl ether derivative are shown in Fig. 1B. The retention time and mass spectrum of the major component of dDHAE-trimethylsilyl ether derivative were same as those of 4-hydroxyvaleric acid ethyl ester-trimethylsilyl ether derivative. Therefore, we concluded that the major component of dDHAE was 4-hydroxyvaleric acid ethyl ester.

The DNA-breaking activity of DHAE and dDHAE was examined by incubating the supercoiled plasmid pGEM®-3Zf(+) DNA with DHAE and

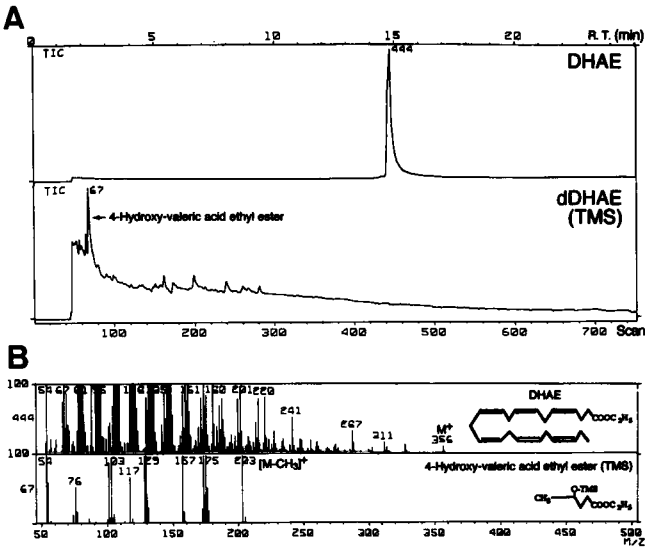

Fig. 1. (A) Total ion chromatogram (TIC) of DHAE and dDHAE. dDHAE was obtained from DHAE by UV irradiation for 60 min. TIC of trimethylsilyl ether (TMS) derivatives of dDHAE shows that a major component with short retention time (scan number 67 of TIC) occures among many peaks. (B) Mass spectra of DHAE (scan number 444 of TIC) and a TMS derivative of the major component of dDHAE (scan number 67 of TIC). The major component was identified as 4-hydroxyvaleric acid ethyl ester, compared with the retention time and mass spectrum of the authentic compound.

dDHAE at 37°C for 3, 9 and 15 hours (Fig. 2A and B). DHAE at a concentration of 100 mg/ml (final concentration of the reaction mixture) cleaved single strands of supercoiled pGEM®-3Zf(+) DNA after a 15 hour incubation. Thus, a large portion of a supercoiled form of DNA (Form I) was transformed into an open circular relaxed form (Form II). However, DHAE at the lower concentrations (0.1, 1 and 5 mg/ml) did not cleave a single-strand even after a 15 hour incubation (data not shown). The dDHAE at a concentration of 100 mg/ml completely destroyed the DNA molecules when incubated for more than 3 hours, and it still cleaved single-strands at a concentration of 5 mg/ml. When incubated with dDHAE (5 mg/ml) for 15 hours, a large portion of supercoiled DNA (Form I) was transformed into open circular relaxed (Form II) and linear (Form III) forms. dDHAE at the lower concentrations (0.1 and 1 mg/ml) did not cleave single-strands even after

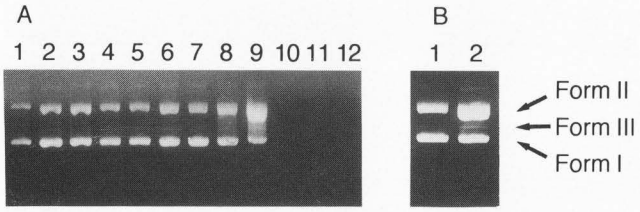

Fig. 2. Agarose gel electrophoresis of supercoiled pGEM®-3Zf(+) DNA exposed with DHAE and dDHAE. (A) Electrophoresis of supercoiled pGEM®-3Zf(+) DNA incubated at 37°C for 3, 9 and 15 hours (lane 1, 2, 3), the DNA incubated with vehicle (ethanol) at 37°C for 3, 9 and 15 hours (lane 4, 5, 6), the DNA incubated with DHAE (100 mg/ml) at 37°C for 3, 9 and 15 hours (lane 7, 8, 9), and the DNA incubated with dDHAE (100 mg/ml) at 37°C for 3, 9 and 15 hours (lane 10, 11, 12). (B) Electrophoresis of supercoiled pGEM®-3Zf(+) DNA incubated with dDHAE (1 mg/ml) at 37°C for 15 hours (lane 1) and the DNA incubated with dDHAE (5 mg/ml) at 37°C for 15 hours (lane 2).

a 15 hours incubation (data not shown).

The effects of DHAE and dDHAE on the growth of HSC-4 cells are shown in Fig. 3. We confirmed the incorporation of external DHAE into cells using GC-MS, although we failed to detect the incorporation of external dDHAE into cells (data not shown). The growth of HSC-4 cells was inhibited by DHAE or dDHAE. The number of adherent cells decreased during the incubation at DHAE or dDHAE concentrations above 10 μg/ml (final concentration of the medium) (Fig. 3A and B). The survival rate of the cells incubated with DHAE or dDHAE was dependent on the concentrations of DHAE or dDHAE (Fig. 3C and D). The lethal effects of DHAE and dDHAE on HSC-4 cell line were quite similar.

The results of a morphological study are shown in Fig. 4. The HSC-4 cells incubated in presence of only vehicle (ethanol) for 24 hours were morphologically intact (Fig. 4B). They were clustered close together, and their shape was angular. However, the cells incubated in the presence of 50 μg/ml of DHAE or dDHAE concentration for 24 hours showed striking morphological changes, becoming sparse and round (Fig. 4C and D).

DISCUSSION

We identified the major component of dDHAE as 4-hydroxyvaleric acid ethyl ester, which has never been reported as a secondary product of DHA and

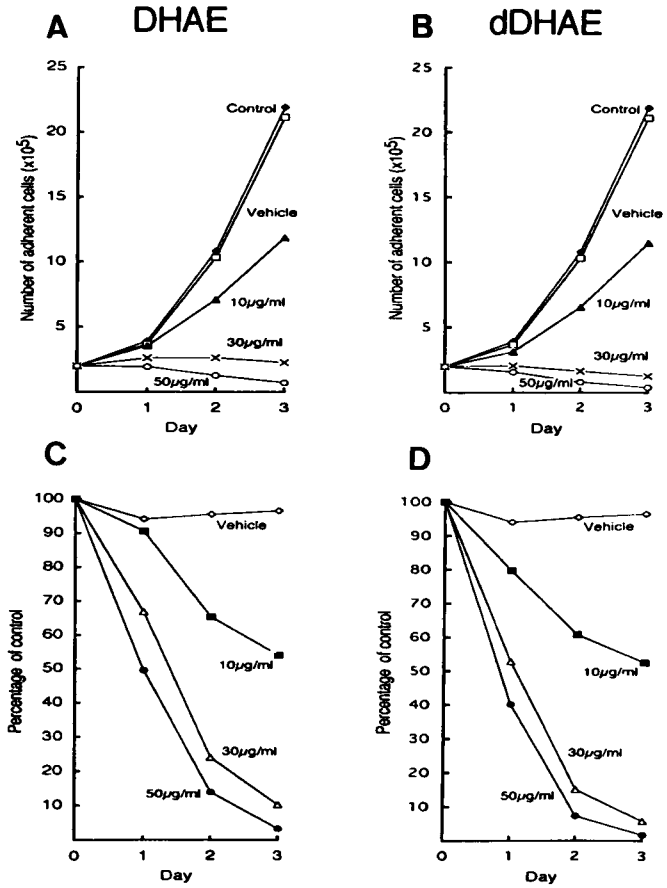

Fig. 3. Cytotoxicity of DHAE and dDHAE on the HSC-4 cells. (A)(B) Numbers of the adherent cells incubated with DHAE and dDHAE, respectively. (C)(D) Survival curves of the cells incubated with DHAE and dDHAE, respectively.

DHAE. The overwhelming majority of dDHAE was 4-hydroxyvaleric acid ethyl ester, although there were many peaks on TIC of dDHAE-trimethylsilyl ether derivative as shown in Fig. 1A. The structure of 4-hydroxyvaleric acid is similar to a hydrolysis product of 4-hydroxy-5-methyl-3(2H)-furanone which has DNA-breaking activity and mutagenicity.[7] The fact suggested that 4-hydroxyvaleric acid has DNA-breaking activity and mutagenicity.

We also demonstrated that DHAE and dDHAE not only inhibited the

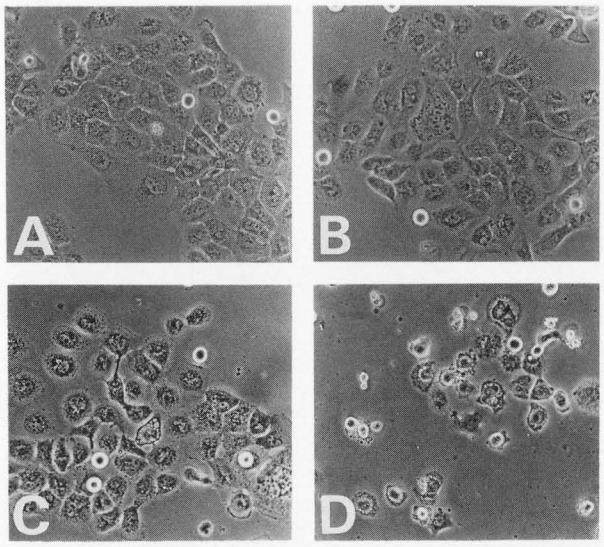

Fig. 4. Morphological features of HSC-4 cells incubated with DHAE and dDHAE at a concentration of 50 μg/ml for 24 hours. (A) Control cells. (B) Cells incubated with vehicle (ethanol). (C) (D) Cells incubated with DHAE and dDHAE, respectively.

proliferation of HSC-4 cells, but also led them to death. Many effects of PUFAs on cells have been reported. The effects can be divided into two classes. One is based on the bioactivities of PUFAs and their metabolites, including prostanoids.[8,9] Recently, an interesting bioactivity of PUFAs, ability to regulate the expression of genes by binding to fatty acid responsive factors, has been reported.[10] The other is based on the radicals derived from PUFAs and their metabolites.[11] PUFAs at appropriate concentrations are tumoricidal *in vitro*.[12,13] The action of PUFAs can be blocked by antioxidants, suggesting that free radicals or lipid peroxidation play a role.[12] In some tumor cell lines, the action of PUFA can also be blocked by cyclo-oxygenase and lipoxygenase inhibitors.[13] These findings indicated that free radicals are generated during PUFA metabolism. Metabolites or degradation products of PUFAs can generate free radicals, causing molecular damage to cellular structures. We suppose that the death of HSC-4 cells by DHAE was due to the cytotoxic action of radicals that originated from DHAE.[12,13] Although the role of the major component of dDHAE, 4-hydroxyvaleric acid ethyl ester, remains to be clarified, we speculate that radicals were also ultimate mediators

of the cytotoxic action of dDHAE.

Our results showed that DHAE was cytotoxic, as described above. Das et al. have also reported that DHA is highly cytotoxic to vincristine-sensitive and resistant human cervical carcinoma cells.[14] On the other hand, Shiina et al. reported that DHA inhibits the proliferation of vascular smooth muscle cells in a non-cytotoxic manner.[15] Their cells showed no morphological changes and normal protein synthesis. These results indicate that though free radicals are the mediators of the anti-proliferative action of DHA, the resistance to radicals, namely scavenging ability, is variable in different types of cells.

There was a large difference in concentrations of DHAE and dDHAE between the DNA-breaking activity and cytotoxicity. The DNA-breaking activity reflects only the chemical attack of the compound against the DNA structure. The cytotoxicity reflects all the harmful effects on proliferation of the cells. The cell proliferation are regulated by many factors in complexity, and ready to be inhibited, whatever the damage to the cellular systems may be. Therefore, cytotoxicity test is much more sensitive to the damage to the cells than DNA-breaking activity is.

We expected that the compounds with greater DNA-breaking activity were more cytotoxic. However, the fact is that the lethal effects of DHAE and its degradation products, dDHAE, on the HSC-4 cell line were quite similar, although the DNA-breaking activity of dDHAE was much greater than that of DHAE. This should be more closely examined. One plausible explanation is as follows: DHAE in the reaction mixture of the DNA-breaking test was little oxidized and generated a limited number of radicals during the short incubation time. Therefore, DHAE had a less potent DNA-breaking activity. On the contrary, dDHAE itself contained many radicals and had a very potent DNA-breaking activity in the reaction mixture. That may be why the DNA-breaking activity of dDHAE was much greater than that of DHAE. With the HSC-4 cell line *in vitro*, this was not so. The external DHA derived from DHAE and incorporated into the cell, was immediately oxidized/metabolized and generated many secondary products of peroxidation and finally many radicals during culture. In the cells incubated with DHAE and dDHAE at the same concentration, almost the same number of radicals may have been generated. This may be why the lethal effects of DHAE and dDHAE on HSC-4 cell line were quite similar.

In conclusion, DHAE and its degradation products showed DNA-breaking activity and cytotoxicity. Our results suggested that DHAE becomes

cytotoxic after undergoing oxidization and metabolism in the cell.

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