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Lipid Peroxidation of a Human Hepatoma Cell Line (HepG2) after Incorporation of Linoleic Acid, Arachidonic Acid, and Docosahexaenoic Acid

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Lipid peroxidation of human hepatoma cell line, HepG2, after incorporation of linoleic acid (LA), arachidonic acid (AA), and docosahexaenoic acid (DHA) was measured with a fluorescent probe and gas chromatography–mass spectrometry (GC–MS) analysis. The analysis with a fluorescent probe showed that incorporation of each polyunsaturated fatty acid (PUFA) enhanced the cellular lipid peroxidation level, but there was little difference in the effect of LA, AA, or DHA on the enhancement of cellular lipid peroxidation. The fluorescent analysis also showed that the addition of H₂O₂ (0.5 mM) enhanced the cellular lipid peroxidation levels in LA and AA supplemented cells as compared with those without H₂O₂. However, the enhancement of lipid peroxidation by H₂O₂ was not observed in DHA-supplemented cells. The same result was obtained in the GC–MS analysis of total amounts of monohydroperoxides (MHP) formed in the cellular phospholipid oxidation. In this case, the main source for MHP was LA in LA-, AA-, and DHA-supplemented cells. A significant amount of AA–MHP and a small amount of DHA–MHP were observed in AA- and DHA-supplemented cells respectively. GC–MS analysis also indicated the specific positional distribution of DHA–MHP isomers. The isomers were formed only by hydrogen abstraction at the C-18 (16-MHP + 20-MHP; 46.5%), C-6 (4-MHP + 8-MHP; 38.5%), and C-12 (10-MHP + 14-MHP; 15.1%) positions, but not at the C-9 or C-15 positions.

Key words: cellular lipid peroxidation; H₂O₂; isomeric distribution of monohydroperoxide; docosahexaenoic acid (DHA) conformation

In the course of investigations of the physiological effects of polyunsaturated fatty acids (PUFAs), the oxidation of PUFAs in cell membranes has received

considerable attention because of its possible contribution to potential damage to biological systems. The susceptibility of each PUFA to oxidation is linearly proportional to the number of bisallylic hydrogens (the degree of unsaturation) in the molecule.¹⁾ Hence, a high content of highly unsaturated fatty acid such as arachidonic acid (20:4n-6; AA) or docosahexaenoic acid (22:6n-3; DHA) in cell membranes may increase oxidative stress on biological systems. On the other hand, we have reported that the oxidative stability of PUFAs in an aqueous system was quite different from that in the bulk phase. In aqueous micelles, stability increased with increasing degree of unsaturation.^{2,3)} In the case of liposomes, the degree of unsaturation had little effect on the stability of phosphatidylcholine (PC) containing linoleic acid (LA), AA, and DHA respectively.⁴⁾ This characteristic oxidative stability of PUFA in aqueous systems is probably due to the different conformation of interface or PC bilayers composed of different PUFAs. These results suggest that the oxidative stability of lipid bilayers in biological membranes might be different from that in the bulk phase or in organic solution.

Kubo *et al.*⁵⁾ reported that DHA ingestion did not increase lipid peroxides to the level expected from the peroxidizability index of the tissue total lipids. Especially, in the brain and testis, lipid peroxide levels decreased when DHA was given to animals. Ando *et al.*⁶⁾ also examined the effects of fish oil on lipid peroxidation of rat organs and found that levels of phospholipid hydroperoxides and thiobarbituric acid-reactive substances (TBARS) in rat organs fed a fish oil diet were similar to those of the safflower-oil diet group. Wander and Du⁷⁾ measured plasma lipid peroxidation after supplementation with eicosapentaenoic acid (EPA) and DHA from fish oil and tocopherol in postmenopausal women. They found that neither plasma TBARS

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; DMEM, Dulbecco's modified Eagle's medium; DPPP, diphenyl-1-pyrenylphosphine; EPA, eicosapentaenoic acid; FBS, fetal bovine serum; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; LA, linoleic acid; MHP, monohydroperoxides; NL, neutral lipids; PBS, phosphate-buffered saline; PL, phospholipids; PUFA, polyunsaturated fatty acid; TBARS, thiobarbituric acid-reactive substances; TL, total lipids; TMS, trimethylsilyl

concentration nor protein oxidation changed after fish oil supplementation. These *in vivo* results suggest a difference in the oxidative stability of PUFA between biological systems and the bulk phase.

In this paper, we report the lipid peroxidation of a human hepatoma cell line, HepG2, after incorporation of LA, AA, and DHA into the cell. This study provides information on the peroxidation of PUFAs such as AA and DHA in biological systems.

Materials and Methods

Materials. LA, AA, and DHA (purity, >99%) were purchased from Nu-Chek-Prep (Elysian, MN). Each PUFA gave only a single spot by thin-layer chromatography,⁸⁾ and none contained tocopherols or peroxides as observed by high-pressure liquid chromatography⁹⁾ or by the determination of peroxide value.¹⁰⁾ Fetal bovine serum (FBS), diphenyl-1-pyrenylphosphine (DPPP), methyl 3-hydroxypentadecanoic acid, *N,O*-bis(trimethylsilyl)acetamide, and H₂O₂ solution were obtained from Wako Pure Chemical (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) was obtained from Nissui Pharmaceutical (Tokyo, Japan). Penicillin-streptomycin solution was from Sigma (St. Louis, MO).

Cell culture. HepG2 (hepatoma) were obtained from Riken Gene Bank (Tsukuba, Japan). Cells (5×10^4 cells/ml) were preincubated in DMEM medium supplemented with 10% FBS, 0.12% NaHCO₃, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. They were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO₂.

Lipid separation and analysis of fatty acid composition. Cells preincubated as described above were seeded at a density of 5×10^4 cells/dish (150 mm) and cultured in 10 ml medium/dish for 48 h. Miyazawa *et al.*¹¹⁾ reported that LA (20 μM) was incorporated into HepG2 cells. Obermeier *et al.*¹²⁾ reported the incorporation of AA, EPA, and DHA into U937 cells, and steady-state levels of incorporation of these PUFAs were obtained after 8 h incubation. Hence, in the present study, each PUFA in ethanol (10 μl) was added to the culture at a final concentration of 25 μM. After 24 h incubation, cells were washed twice with Ca²⁺ and Mg²⁺ free phosphate-buffered saline (PBS(-)), and total lipids (TL) were extracted with chloroform/methanol (2:1, vol/vol). TL was separated on a Sep-Pak Silica Cartridge column (Waters, Milford, MA). Lipid fractions were sequentially eluted using chloroform and acetone for neutral lipids (NL) and methanol for phospholipids (PL). Each lipid was transmethylated with 0.5 M CH₃ONa in MeOH by heating in a sealed tube at 60–70 °C for 30 min under nitrogen. The fatty acid methyl esters were extracted with hexane. The extract was washed with water, dried over anhydrous sodium sulfate, concentrated *in vacuo*, purified by silicic acid column chromatography, and

then put through to gas chromatography (GC). GC analysis was done on a Shimadzu GC-14B equipped with a flame-ionization detector and a capillary column (Omegawax 320, 30 m × 0.32 mm i.d.; Supelco, Bellefonte, PA).

Determination of cell proliferation by WST-1 assay. Cells preincubated as described above were seeded at a density of 5×10^4 cells/well in 96-well microplates and cultured in 100 μl medium/well for 48 h. Each PUFA was dissolved in 1% dimethyl sulfoxide solution (10 μl) and then added to the culture at a final concentration of 25 μM. After 24 h incubation, different concentrations of H₂O₂ solution (10 μl) were added to the culture and incubated for 21 h. The percentage of viability of HepG2 cells was determined by WST-1 assay,¹³⁾ a modification of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye reduction (MTT) assay based on the terazolium salt/formazan system. After incubation, 10 μl of WST-1 solution with 1-methoxy PMS solution was added to each well, and the plate was incubated for a further 3 h. Cell viability was then measured spectrophotometrically (Microplate reader, Emax; Molecular Devices, Sunnyvale, CA), and expressed as a percentage of the viability obtained in control cultures, which were incubated without H₂O₂ solution. H₂O₂ showed a cytotoxic effect on the growth of HepG2 cells with or without the addition of PUFA. This effect was dose-dependent, and the viability decreased to 70–80% at 1 mM H₂O₂. But, there was a little difference in the viability between control cells and cells treated with 0.5 mM H₂O₂.

Estimation of lipid peroxidation of cells using a fluorescence probe. Lipid peroxidation in the cell was estimated by the fluorescent method with DPPP.^{14,15)} Cells were seeded at a density of 5×10^4 cells/dish (100 mm) and cultured in 10 ml medium/dish for 48 h. Each PUFA in ethanol (10 μl) was added to the culture at a concentration of 25 μM. After 24 h incubation, cells were washed three times with PBS(-). The cell suspension was incubated with 50 μM DPPP solution (10 ml) for 15 min at 37 °C. DPPP was dissolved in dimethylsulfoxide and dispersed in the PBS. Then the cells were washed twice with the buffer. The DPPP-containing cells were cultured in 10 ml medium/dish with or without 100 μl H₂O₂ (0.5 mM). After incubating for 24 h at 37 °C, the cells were washed three times with PBS(-) and resuspended in PBS(-), and the fluorescence intensities of the samples were measured with a Hitachi F-200 spectrofluorophotometer with excitation and emission wavelengths of 351 and 380 nm respectively. During the labeling procedure by DPPP, cell suspension was handled in shaded tubes and kept in the dark.

Estimation of hydroperoxides formed in the oxidation of PL of cells by GC-mass spectrometry (MS) analysis.

Cells were seeded at a density of 5×10^4 cells/dish (150 mm) and cultured in 10 ml medium/dish for 48 h. Each PUFA in ethanol (10 μ l) was added to the culture at a concentration of 25 μ M. After 24 h incubation, the cells were cultured in 10 ml medium/dish (105 mm) with 100 μ l H_2O_2 (0.5 mM). After 24 h incubation, the cells were washed twice with PBS(-) and TL was extracted with chloroform/methanol (2:1, vol/vol). TL was separated on a Sep-Pak Silica Cartridge column into NL and PL, as described above.

The PL was dissolved in chloroform and 250 pmol of methyl-3-hydroxypentadecanoate was added to the solution as an internal standard, and the mixture was dissolved into 5 ml of methanol and hydrogenated by bubbling with hydrogen gas (5 ml/min) for 1 h in the presence of palladium black. The solution was then filtrated to remove the palladium black and dried over a nitrogen gas stream. The residue was dissolved into 1.0 M sodium methoxide methanol solution (1 ml) under nitrogen and heated at 60 °C for 1 h to convert the PL into methyl esters. After cooling, it was washed with 2 ml of water and extracted twice with 2 ml of hexane. The extract was washed with 3 ml of water three times, and sodium sulfate was added for removal of water. The sodium sulfate was filtered and the filtrate was dried. The resultant methyl ester was subjected to GC-MS analysis. The lipids obtained after the reactions were trimethylsilylated and subjected to GC-MS according to the method reported previously,¹⁶⁾ with modifications.

The hydrogenated lipid samples were dissolved into 100 μ l of *N,O*-bis(trimethylsilyl)acetamide acetonitrile solution and then heated at 80 °C for 1 h for trimethylsilylation. The resulting solution (1 μ l) was introduced directly into a QP-5000 GC-MS system (Shimadzu, Kyoto, Japan) by the splitless injection method, using electron impact ionization at 70 eV. A capillary column (0.25 mm i.d \times 30 m) of DB-1 (J & W Scientific, CA) was used for GC separation. The column temperature was held at 120 °C for 1 min, raised to 150 °C at a rate of 5 °C/min, then to 250 °C at a rate of 10 °C/min, and finally held at 250 °C for 10 min. Helium gas was used as the carrier at a flow rate of 2.8 ml/min. The area of the peaks in selected ion monitoring spectra were normalized by the area of the prominent peak of the methyl 3-trimethylsilyloxyoctadecanoate (10.3 min, *m/e* = 329 ($[M-CH_3]^+$), derived from 3-hydroxypentadecanoate for calculation of the amount of each hydrogenated oxidation product.

When trimethylsilyl (TMS)-derivatives of hydroxy fatty acid ethyl or methyl esters are subjected to mass spectrometry, one of the two C-C bonds next to the carbon attached to trimethylsiloxy group is cleaved at ionization. For example, methyl 9-trimethylsilyloxyoctadecanoate derived from one of the monohydroperoxide (MHP) isomers obtained by the oxidation of linoleate gives two radical cations of $[CH(OTMS)C_8H_{16}CH_3]^+$ (*m/e* 229) and $[CH(OTMS)C_7H_{14}COOC_2H_5]^+$ (*m/e* 243) by cleavage of the C8-C9 and C9-C10 bonds

respectively. Each MHP isomer can be distinguished by monitoring these specific fragments.¹⁷⁻²⁰⁾

Quantitative comparison of TMS derivatives from mono-hydroxy compounds (MH) was done using their peak areas on total ion monitoring by the GC-MS. Each TMS-derivative was identified by its retention time on GC and mass number of $[M^+]$.²¹⁾

Statistical analysis. Data are expressed as means \pm SD. Data were analyzed by one-way analysis of variance, and the significant differences among means were inspected with Tukey's test at the level of $P < 0.01$.

Results

Incorporation of LA, AA, and DHA into cellular lipids of HepG2 cells

The addition of 25 μ M LA, AA, or DHA to the culture medium and incubation for 24 h was followed by remarkable incorporation of the respective PUFAs into cellular TL, while untreated HepG2 cells (control) contained relatively low levels of LA (18:2n-6), AA (20:4n-6), or DHA (22:6n-3) (Table 1). The increase in LA, AA, or DHA was compensated by a decrease in monoenoic fatty acids such as 18:1n-9 and 18:1n-7. The same change in fatty acid composition was also found in cellular NL (Table 2). The content of LA, AA, or DHA in cellular NL after the addition of each PUFA was significantly higher than that in the NL of the control cells. The content of each PUFA in the cellular PL after the addition of each PUFA was also significantly higher than that in the PL of the control cells (Table 3).

Cellular lipid peroxidation

The cells treated with or without a PUFA for 24 h were incubated with DPPP for 15 min. The DPPP-containing cells were further incubated for 24 h with or without H_2O_2 . Then the cellular lipid peroxidation level was determined by measurement of DPPP oxide, which is formed by the reaction of DPPP with hydroperoxide. In the absence of H_2O_2 , the level of cellular lipid

Table 1. Incorporation of Each PUFA into the TL of Cells Incubated with LA, AA, and DHA for 24 h

| Fatty acid (wt %) | Cell | | | |
|----------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| | Control | + LA | + AA | + DHA |
| 16:0 | 25.1 \pm 0.4 ^a | 23.7 \pm 0.2 ^a | 23.3 \pm 1.0 ^a | 25.3 \pm 0.6 ^a |
| 18:0 | 5.8 \pm 0.4 ^a | 5.7 \pm 0.2 ^a | 6.9 \pm 0.6 ^a | 7.1 \pm 0.1 ^a |
| 16:1n-7 | 7.2 \pm 0.1 ^a | 4.4 \pm 0.0 ^b | 3.5 \pm 0.2 ^c | 4.6 \pm 0.1 ^b |
| 18:1n-7 | 12.9 \pm 0.2 ^a | 9.4 \pm 0.2 ^b | 7.9 \pm 0.7 ^c | 8.1 \pm 0.1 ^{bc} |
| 18:1n-9 | 22.9 \pm 0.4 ^a | 17.0 \pm 0.2 ^b | 15.8 \pm 1.6 ^b | 17.2 \pm 0.4 ^b |
| 18:2n-6 | 2.6 \pm 0.1 ^a | 15.2 \pm 0.5 ^b | 2.2 \pm 0.6 ^a | 1.9 \pm 0.0 ^a |
| 20:4n-6 | 4.9 \pm 0.2 ^a | 5.0 \pm 0.1 ^a | 20.3 \pm 3.6 ^b | 4.1 \pm 0.1 ^a |
| 22:6n-3 | 3.3 \pm 0.1 ^a | 2.6 \pm 0.1 ^a | 2.9 \pm 0.0 ^a | 17.3 \pm 1.2 ^b |

Data are expressed as mean \pm SD (n = 3).

Values not sharing a common superscript are significantly different at $P < 0.01$.

Table 2. Incorporation of Each PUFA into the NL of Cells Incubated with LA, AA, and DHA for 24 h

| Fatty acid (wt %) | Cell | | | |
|-------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| | Control | + LA | + AA | + DHA |
| 16:0 | 19.3 ± 2.1 ^a | 18.3 ± 1.0 ^a | 19.7 ± 1.0 ^a | 21.8 ± 0.8 ^a |
| 18:0 | 4.4 ± 1.3 ^a | 3.3 ± 0.1 ^a | 3.4 ± 0.6 ^a | 3.2 ± 0.2 ^a |
| 16:1n-7 | 3.5 ± 0.4 ^{ab} | 3.0 ± 0.2 ^a | 3.0 ± 0.1 ^{ab} | 4.0 ± 0.2 ^b |
| 18:1n-7 | 8.5 ± 0.8 ^a | 6.6 ± 0.2 ^b | 6.4 ± 0.7 ^b | 7.8 ± 0.3 ^{ab} |
| 18:1n-9 | 21.2 ± 2.0 ^a | 17.2 ± 0.6 ^a | 17.5 ± 1.6 ^a | 19.3 ± 0.6 ^a |
| 18:2n-6 | 1.6 ± 0.3 ^a | 22.5 ± 1.4 ^b | 2.3 ± 0.6 ^a | 1.1 ± 0.0 ^a |
| 20:4n-6 | 0.8 ± 0.1 ^a | 1.7 ± 0.1 ^a | 14.1 ± 3.6 ^b | 0.6 ± 0.1 ^a |
| 22:6n-3 | 2.1 ± 0.1 ^a | 2.4 ± 0.2 ^a | 2.7 ± 0.0 ^a | 23.1 ± 2.6 ^b |

Data are expressed as mean ± SD (n = 3).

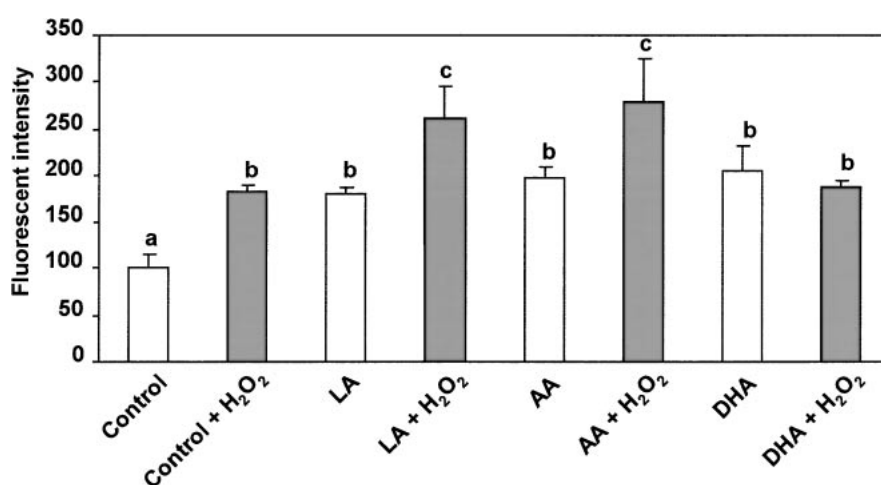
Values not sharing a common superscript are significantly different at $P < 0.01$.

Table 3. Incorporation of Each PUFA into the PL of Cells Incubated with LA, AA, and DHA for 24 h

| Fatty acid (wt %) | Cell | | | |
|-------------------|-------------------------|-------------------------|-------------------------|--------------------------|
| | Control | + LA | + AA | + DHA |
| 16:0 | 22.2 ± 1.0 ^a | 22.4 ± 0.7 ^a | 22.1 ± 0.8 ^a | 23.2 ± 0.7 ^a |
| 18:0 | 9.0 ± 0.2 ^a | 11.2 ± 0.1 ^b | 12.4 ± 0.1 ^c | 11.8 ± 0.2 ^{bc} |
| 16:1n-7 | 5.4 ± 0.2 ^a | 3.6 ± 0.2 ^b | 2.9 ± 0.3 ^c | 4.1 ± 0.4 ^b |
| 18:1n-7 | 9.4 ± 0.1 ^a | 7.5 ± 0.1 ^b | 7.0 ± 0.6 ^b | 7.3 ± 0.1 ^b |
| 18:1n-9 | 16.7 ± 0.1 ^a | 13.1 ± 0.1 ^b | 11.4 ± 0.7 ^c | 13.8 ± 0.1 ^b |
| 18:2n-6 | 2.8 ± 0.1 ^a | 12.2 ± 0.1 ^b | 1.5 ± 0.1 ^a | 3.2 ± 1.0 ^a |
| 20:4n-6 | 9.8 ± 0.1 ^a | 10.9 ± 0.1 ^a | 19.7 ± 0.3 ^b | 8.3 ± 0.5 ^c |
| 22:6n-3 | 4.9 ± 0.0 ^a | 3.9 ± 0.1 ^{ab} | 3.2 ± 0.2 ^a | 10.5 ± 0.8 ^c |

Data are expressed as mean ± SD (n = 3).

Values not sharing a common superscript are significantly different at $P < 0.01$.

**Fig. 1.** Formation of MHP in HepG2 Cells Treated with or without H₂O₂.

Values not sharing a common superscript are significantly different at $P < 0.01$. Values are mean ± S.D. (n = 3).

peroxidation treated with each PUFA was significantly higher than that of untreated cells (the control cells) (Fig. 1). However, there was little difference in the peroxidation level in LA-, AA-, and DHA-supplemented cells. On the other hand, the addition of H₂O₂ enhanced the peroxidation level of the control, LA- and AA-supplemented cells as compared with those without H₂O₂ treatment. But, little effect of H₂O₂ was observed on DHA supplemented cells.

MHP formation in the cellular PL

The amount of MHP formed in the cellular PL oxidation with or without H₂O₂ was measured by GC-MS analysis. Identification and quantification of each positional MHP isomer was carried out after conversion of MHP to the corresponding monohydroxy derivative. As shown in Table 4, the main MHP was LA-MHP in control cells and LA-, AA-, and DHA-supplemented cells with or without H₂O₂. AA-MHP was found as a major MHP in the AA supplemented cells, but the amount of AA-MHP was lower than that of LA-MHP. DHA-MHP was not found in control cells or LA- or

AA-supplemented cells, but a small amount of DHA-MHP was detected in the DHA supplemented cells. The highest total amount of MHP was shown by AA-supplemented cells, followed by LA-supplemented cells, DHA-supplemented cells, and control cells without H₂O₂. In the case of H₂O₂ treatment, AA-supplemented cells showed the highest amount of total MHP, followed by LA-supplemented cells, control cells, and DHA-supplemented cells. This order was almost the same as that of the cellular peroxidation level after treatment with H₂O₂ as measured by fluorescent analysis (Fig. 1).

Positional distribution of MHP isomers in the oxidation of cellular PL

The free-radical oxidation of PUFAs starts from hydrogen abstraction at a bis-allylic methylene group, which produces a hybrid pentadienyl radical. This radical reacts with oxygen, giving two kinds of MHPs with a conjugated diene. In LA, hydrogen abstraction occurs at the carbon-11 position, which results in production of a pentadienyl radical between carbon-9 and carbon-13. Then the radical reacts at either end with

Table 4. Amounts of MHP Formed in the Oxidation of Cellular PL with or without H₂O₂

| | Cell | Amounts of MHP (μmol/mmol lipid) | | | |
|---------------------------------------|---------|----------------------------------|-------------|-------------|-------------|
| | | LA-MHP | AA-MHP | DHA-MHP | Total MHP |
| Without H ₂ O ₂ | Control | 1.80 ± 0.43 | 0.15 ± 0.05 | ND | 1.95 ± 0.48 |
| | + LA | 3.06 ± 0.40 | 0.17 ± 0.08 | ND | 3.23 ± 0.35 |
| | + AA | 2.17 ± 0.35 | 1.23 ± 0.11 | ND | 3.40 ± 0.29 |
| | + DHA | 2.37 ± 0.31 | 0.21 ± 0.06 | 0.36 ± 0.05 | 2.93 ± 0.20 |
| With H ₂ O ₂ | Control | 3.10 ± 0.62 | 0.32 ± 0.09 | ND | 3.42 ± 0.71 |
| | + LA | 3.50 ± 0.46 | 0.17 ± 0.06 | ND | 3.67 ± 0.41 |
| | + AA | 2.47 ± 0.47 | 1.55 ± 0.57 | ND | 4.02 ± 1.03 |
| | + DHA | 2.27 ± 0.47 | 0.20 ± 0.09 | 0.33 ± 0.10 | 2.79 ± 0.65 |

Data are expressed as mean ± SD (n = 3).
ND, not detected.

Table 5. Isomeric Distribution of LA-MHP Isomers in Oxidation of Cellular PL after Treatment with H₂O₂

| PUFA added | Isomer distribution (%) | |
|----------------|-------------------------|-------------------------|
| | 9-MHP | 13-MHP |
| None (control) | 50.3 ± 2.1 ^a | 49.7 ± 2.1 ^a |
| + LA | 49.7 ± 1.5 ^a | 50.3 ± 1.5 ^a |
| + AA | 50.7 ± 1.5 ^a | 49.3 ± 1.5 ^a |
| + DHA | 50.0 ± 1.7 ^a | 50.0 ± 1.7 ^a |

Data are expressed as mean ± SD (n = 3).
Values not sharing a common superscript are significantly different at $P < 0.01$.

oxygen to produce a mixture of 9- and 13-MHP. Since AA and DHA have three and five bis-allylic methylene groups respectively, there are several possible positions for hydrogen abstraction: carbon 7, 10, 13 for AA, and carbon-6, 9, 12, 15, 18 for DHA. Since oxygen can attack carbon atoms at either end of the pentadienyl radical, the resulting the mixture of MHP isomers were

those with hydroperoxide substitution on carbons at 5, 9, 8, 12, 11, and 15 for AA, and at 4, 8, 7, 11, 10, 14, 13, 17, 16, and 20 for DHA.

LA- and AA-MHP isomers were detected in the cellular PL from all cells treated with H₂O₂ (Tables 5 and 6). On the other hand, some limited DHA-MHP isomers were detected only in DHA-supplemented cells (Table 7). As shown in Table 5, there was no significant difference in the distribution pattern of the two kinds of LA-MHP isomers in any case. On the other hand, the highest percentage was found for 15-MHP in AA oxidation, followed by the 5-, 12-, 8-, 9-, and 11-MHP isomers respectively (Table 6). In DHA-MHP (Table 7), the distribution of MHP derived from hydrogen abstraction at carbon-18 (16-MHP + 20-MHP) was the highest (46.5%), followed by those at carbon-6 (4-MHP + 8-MHP; 38.5%) and carbon-12 (10-MHP + 14-MHP; 15.1%). However, MHP derived from hydrogen abstraction at carbon-9 (7-MHP + 11-MHP) and those at carbon-15 (13-MHP + 17-MHP) were not

Table 6. Isomeric Distribution of AA-MHP Isomers in Oxidation of Cellular PL after Treatment with H₂O₂

| PUFA added | Isomer distribution (%) | | | | | |
|----------------|---------------------------|---------------------------|--------------------------|-------------------------|--------------------------|--------------------------|
| | 5-MHP | 8-MHP | 9-MHP | 11-MHP | 12-MHP | 15-MHP |
| None (control) | 23.3 ± 2.8 ^{ad} | 11.4 ± 2.1 ^{bc} | 9.7 ± 1.5 ^{bc} | 4.1 ± 2.1 ^c | 18.7 ± 3.0 ^{ab} | 32.7 ± 3.0 ^d |
| + LA | 25.3 ± 1.4 ^a | 13.4 ± 2.0 ^b | 11.0 ± 1.5 ^{bc} | 3.8 ± 2.0 ^c | 15.6 ± 1.7 ^b | 30.9 ± 4.5 ^a |
| + AA | 23.7 ± 3.7 ^{ad} | 12.9 ± 2.3 ^{bce} | 6.6 ± 1.3 ^c | 3.4 ± 2.3 ^{bc} | 23.2 ± 3.9 ^{de} | 30.2 ± 3.4 ^{ad} |
| + DHA | 25.2 ± 1.8 ^{ace} | 14.9 ± 2.5 ^{ab} | 5.8 ± 5.1 ^{bc} | 4.0 ± 2.5 ^b | 17.3 ± 3.3 ^{ac} | 32.7 ± 3.6 ^{de} |

Data are expressed as mean ± SD (n = 3).
Values not sharing a common superscript are significantly different at $P < 0.01$.

Table 7. Isomeric Distribution of DHA-MHP Isomers in Oxidation of Cellular PL after Treatment with H₂O₂

| PUFA added | Isomer distribution (%) | | | | | | | | | |
|----------------|-------------------------|-------|-------------------------|-------------------------|--------|--------|------------------------|-------------------------|--------|-------------------------|
| | 4-MHP | 7-MHP | 8-MHP | 10-MHP | 11-MHP | 13-MHP | 14-MHP | 16-MHP | 17-MHP | 20-MHP |
| None (control) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| + LA | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| + AA | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| + DHA | 25.7 ± 2.2 ^a | ND | 12.8 ± 1.6 ^b | 10.2 ± 1.3 ^b | ND | ND | 4.9 ± 4.5 ^b | 24.6 ± 1.5 ^a | ND | 21.9 ± 4.3 ^a |

Data are expressed as mean ± SD (n = 3).
ND, not detected.
Values not sharing a common superscript are significantly different at $P < 0.01$.

detected. The MHP distribution in cellular PL oxidation without H₂O₂ was also carried out. The distribution pattern was almost the same as that in cellular PL oxidation after treatment with H₂O₂.

Discussion

Supplementation with LA, AA, or DHA resulted in their incorporation into cellular TL, NL, and PL (Tables 1–3). Fluorescent analysis showed that the incorporation of PUFA enhanced cellular lipid peroxidation (Fig. 1). Igarashi and Miyazawa¹¹ reported an increase in membrane phospholipid hydroperoxide after incorporation of LA in HepG2 cells. The fluorescent analysis of lipid peroxidation was based on the oxidation of DPPP to DPPP oxide by organic peroxides present in the cell membranes.^{14,15} Therefore, the result in Fig. 1 represents the peroxidation level of cell membrane PL. The average number of bis-allylic positions per 1 mol of fatty acid in cellular PL can be calculated from the fatty acid composition (Table 3) to be 0.57 for control cells, 0.64 for LA supplemented cells, 0.77 for AA supplemented cells, and 0.81 for DHA supplemented cells. It is known that the oxidative stability of PUFA decreases with increasing numbers of bis-allylic positions.¹ Therefore, perhaps the lipid peroxidation level in the cellular PL of DHA supplemented cells was the highest, followed by AA supplemented cells, LA supplemented cells, and control cells. However, as shown in Fig. 1, there was little difference in the lipid peroxidation level in the LA, AA, and DHA supplemented cells.

Figure 1 also shows that the addition of H₂O₂ (0.5 mM) enhanced cellular lipid peroxidation levels in control, LA-, and AA-supplemented cells as compared with those without H₂O₂. The enhancement of the lipid peroxidation level by the addition of H₂O₂ has been also reported in mouse polymorphonuclear leukocytes¹⁴ and in human monocytic leukemia cells (U-937)¹⁵ at a range of 0.1–1.0 mM of H₂O₂. On the other hand, induction of lipid peroxidation by H₂O₂ was not observed in DHA-supplemented cells. The same result was obtained by analysis of total MHP content in the cellular PL oxidation of control, LA-, AA-, and DHA-supplemented cells (Table 4). Table 4 also shows that the main source for MHP was LA in all cases, and a significant amount of AA-MHP was observed only in AA-supplemented cells. A small amount of DHA-MHP was also observed in DHA-supplemented cells, but not in other cells. PUFA containing more than two double bonds, such as AA and DHA, produces further oxidation products from MHP such as hydroperoxy epidioxides.²² The relatively lower contribution of AA and DHA to MHP formation found in Table 4 might be due to the further oxidation of AA-MHP and DHA-MHP.

It is generally accepted that the oxidative stability of PUFA in the bulk phase and in organic solution is inversely proportional to the degree of unsaturation.¹ But, it has been reported that the oxidative stability of

PUFA and its ester in an aqueous environment increased with increasing degrees of unsaturation,^{2,3,23} and that PC containing DHA (PC-DHA) was a little more stable oxidatively than PC-AA and PC-LA in liposomes as a model of biological membrane.⁴ The difference in the oxidative stability of PC in liposomes and in the bulk phase or an organic solvent is due to the specific conformation of PC bilayers in the liposomes.⁴ The order of lipid peroxidation levels of the cellular PLs found in Fig. 1 and Table 4 was almost the same as that in the oxidative stability of PC in liposomes, suggesting that the characteristic cellular lipid peroxidation found in the present study is also correlated with the PUFA conformation in the membrane PL. ¹H- and ¹³C-MAS NMR analysis of DHA-PC liposome^{24–28} showed the possibility of a wide variety of specific conformation of DHA, including back-bended (hairpin-like), helical, and angle-iron conformations. This variety in DHA conformation gives looser packing of the DHA chains. The looser packing of the membrane at the lipid–water interface brings about high water permeability. Molecular dynamics simulation²⁶ also indicates a remarkable overlapping of water molecules with double-bond regions of the DHA chain. The presence of water molecules near the DHA molecule lowers the density of bis-allylic hydrogen and reduces the chain-carrying reaction of lipid peroxidation. The different oxidative stability of DHA in the bulk phase and in liposome or biological membrane lipids might be derived from the specific conformation and high water permeability in the lipid bilayers. The oxidative stability of AA in biological systems might also be influenced by its conformation and physicochemical properties.

Very recently we reported a comparative study on PC oxidation products, mainly positional distribution of MHP isomers, in *t*-BuOH solution and in liposome.²³ Oxidation of PC-LA gave the same distribution of 9- and 13-MHP isomers, both in *t*-BuOH solution and in liposome. With the oxidation of PC-AA and PC-DHA in *t*-BuOH solution, there was little difference in MHP isomeric distributions. However, in PC-AA liposome, the distribution of 9-, 12-, and 15-MHP was much larger than that of 5-, 8-, or 11-MHP.²³ In PC-AA oxidation in liposome, the distribution of MHP, derived from hydrogen abstraction, at C-7 (5-MHP + 9-MHP), at C-10 (8-MHP + 12-MHP), and at C-13 (11-MHP + 15-MHP) were almost the same. These results in PC-AA liposome were very similar to those of AA-MHP isomeric distributions of cellular PL oxidation, except for the higher distribution of 5-MHP in the cells (Table 6). In the case of PC-DHA liposome, the MHP derived from hydrogen abstraction at C-18 (16-MHP + 20-MHP), at C-6 (4-MHP + 8-MHP), at C-12 (10-MHP + 14-MHP), at C-15 (13-MHP + 17-MHP), and at C-9 (7-MHP + 11-MHP) were 37.7%, 29.1%, 17.6%, 8.6% and 8.3% respectively.²³ This tendency was also consistent with the result of DHA-MHP composition of cellular PL in cells supplemented with DHA

(Table 7). In the oxidation of DHA in the cellular PL, DHA-MHP was formed only by abstraction at C-6, C-12, and C-18, but not at the C-9 or C-15 position. This characteristic distribution of DHA-MHP isomers found in the cellular PL oxidation indicates uneven hydrogen abstraction from DHA molecules in the cell membrane lipid, which might also be derived from the specific conformation of the DHA molecule and higher water permeability in the membrane lipids. Hence, further studies are necessary to reveal the relationship between the conformation and physicochemical properties of AA and DHA and their oxidative stability in biological membrane lipids. In addition, it is important to determine the activity of endogenous membrane antioxidants or free radicals on AA and DHA which can take specific conformation in the membrane lipids.

Some reports⁵⁻⁷⁾ have revealed little influence on lipid peroxidation in animals fed DHA or DHA containing fish oil, showing that the oxidative stability of highly unsaturated fatty acids such as DHA in biological systems is different from that in a bulk or organic solution system. The present study gives information for better understanding oxidation in biological systems, but, only HepG2 was used for model cell lines in the present study and concentrations of fatty acid and H₂O₂ were limited. Hence, more information on the effect of DHA and other PUFAs incorporated into various cell lines is needed to elucidate the mechanism for the characteristic oxidative stability of DHA in biological systems.

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