Fluorescence Formation from Esterified Linoleic Acid Hydroperoxides with Amino Compounds

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Polyunsaturated fatty acids esterified to phospholipids, triglycerides and cholesterol esters are important targets for lipid peroxidation, and this leads to production of their peroxides in biological tissues. All linoleic acid hydroperoxides esterified to their backbones yielded fluorescent substances through reaction with 1-aminopentane as a model amino compound in the presence of heme. These fluorescent substances were eluted as major fluorescence peaks (FS-III$_p$, FS-III$_t$ and FS-III$_c$) at different retention times on high-performance liquid chromatography. Each FS-III was hydrolyzed with the respective hydrolytic enzymes, phospholipase A_2, lipase and cholesterol esterase, and was converted to the same fluorescent substance (FS-II) as that formed from methyl linoleate hydroperoxide. The fluorescent substances obtained showed the same fluorescence spectra with excitation and emission maxima at around 350 and 420 nm, respectively. These results indicate that esterified peroxides in phospholipids, triglycerides and cholesterol esters produce esterified fluorescent substances through reaction with amino compounds, resulting in the accumulation of fluorescent substances with age in biological tissues. Moreover, the actions of phospholipase A_2, lipase and cholesterol esterase may lead to elimination of these fluorescent substances from the biological tissues.

Keywords esterified fluorescent substance; hydroperoxide; phosphatidylcholine; triglyceride; cholesterol ester; phospholipase A_2; lipase; cholesterol esterase

Lipid peroxides produced from polyunsaturated fatty acids (PUFA) react with various biological materials, such as amino acids, proteins or deoxyribonucleic acid, causing a wide variety of cell damage. Fluorescent pigments as end-products of lipid peroxidation *in vivo* or *in vitro* have been generally thought to be formed by the reaction of lipid peroxides with amino compounds.[1,2] However, the mechanism of formation of fluorescent substances by lipid peroxides as well as the mechanism of accumulation of fluorescent pigments in aged animal tissues has not yet been clarified.

The degraded compounds produced from hydroperoxides (HPO) of linoleic acid or its methylester (ML) in the presence of heme, metals or ascorbic acid are known to be involved in the formation of fluorescent substances through reaction with amino compounds as a model system *in vitro*.[3–6] In biomembranes, the PUFA moieties are mainly attached to the glycerol backbone of phospholipids. Linoleic acid acylated at the 2-glycerol position in phospholipids yields both 9- and 13-HPO of linoleic acid without being released from their glycerol backbones.[7] We have demonstrated that 1-palmitoyl-2-linoleoyl-phosphatidylcholine (PLPC)-HPO and trilinolein (TL)-HPO produce fluorescent substances exhibiting the same fluorescence spectra as that formed from ML-HPO through reaction with 1-aminopentane (1-AP) as a model amino compound after decomposition by heme.[8] This major fluorescent substance remains attached to the glycerol backbone in PLPC and TL, since it is released by transmethylation to produce the same fluorescent substance (FS-II) as that formed from ML-HPO with 1-AP. Moreover, a fluorescent substance esterified to the glycerol backbone in PLPC was detected on reverse-phase high-performance liquid chromatography (HPLC) and was hydrolyzed with phospholipase A_2.[9] These results suggest that the formation of esterified fluorescent substances in phospholipids may result in the accumulation of fluorescent substances in biological membranes and that phospholipase A_2 may serve to eliminate such fluorescent substances from biological membranes.

Many kinds of PUFA are present as constituents of phospholipids, triglycerides and cholesterol esters in biological tissues, and these PUFA are important targets for lipid peroxidation *in vivo*. In the present study, the esterified fluorescent substances formed from each HPO of PLPC, TL and cholesterol linoleate (CL) with 1-AP in the presence of heme were detected by HPLC, and their hydrolysis with the respective hydrolytic enzymes was investigated as a model system of the fluorescence formation in biological tissues.

Materials and Methods

Materials PLPC, TL, CL, hemin (type I), phospholipase A_2 (porcine pancreas, 830 U/mg protein), lipase (type II), cholesterol esterase (bovine pancreas) and lipoxygenase (type I) were purchased from Sigma Chemical Co. (St. Louis, MO). ML and 1-AP were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo). Heme methyl ester (heme) was prepared by the method of Ortiz de Montellano *et al.*[10]

Preparation of HPO from ML, PLPC, TL and CL The preparation of each HPO from ML, PLPC, TL and CL using lipoxygenase was carried out as described previously.[8] Each HPO was purified by HPLC or thin-layer chromatography (TLC) and was estimated using a molar absorbance value of 24500 at 233 nm.

Fluorescence Formation and Analysis Each HPO (200 nmol) prepared from ML, PLPC, TL and CL was preincubated with heme (2 nmol) in 2 ml of methanol solution at 37 °C for 30 min, and then 1 ml of 1-AP (1 μmol) in methanol solution was added to the preincubation mixture. Fluorescence was developed at 37 °C for 20 h in methanol solution. The fluorescence spectra were measured with a Hitachi MPF-3 fluorescence spectrophotometer, and the intensity was expressed as a percentage of that of a quinine sulfate standard (0.1 μg/ml in 0.1 N H_2SO_4). The total fluorescence intensity of each sample was adjusted to the same fluorescence strength (1000%) by concentration under an N_2 stream, and an aliquot of each sample was subjected to reverse-phase HPLC, performed on a μ-Bondapak FAA column (4.0 × 30 mm, Waters Associates, Milford, MA) with a packed guard column (4 × 50 mm, Unisil C_{18}, Gasukuro Kogyo Inc., Tokyo). Elution was carried out with acetonitrile–water (50 : 50) for the first 10 min and then the acetonitrile concentration was increased to 100% over 20 min at a flow rate of 1.0 ml/min. The fluorescence peaks were monitored at excitation and emission maxima of 350 and 420 nm with a Hitachi 650-10LC fluorescence spectrophotometer. The fluorescent substances were also subjected to TLC (Kieselgel 60, Merck, Darmstadt, FRG) and developed with chloroform–methanol (9 : 1).

Hydrolysis of Fluorescent Substances Hydrolysis of the fluorescent substances formed from PLPC-HPO by phospholipase A_2 was carried out as described previously.[9] Fifty microliters of the fluorescent sample formed from TL-HPO with 1-AP was evaporated under an N_2 stream and was dispersed in 0.5 ml of 10 mM sodium deoxycholate by vortexing. The reaction was carried out at 37 °C for 30 min in 2 ml of 0.1 M Tris–HCl buffer (pH 8.2) containing 5 mM $CaCl_2$ and 1 mg of lipase. Fifty microliters of the fluorescent sample prepared from CL-HPO was also treated under the same conditions in 2 ml of 0.1 M phosphate buffer (pH 6.8) containing 1 mg of cholesterol esterase. The reaction products were extracted with 5 ml of chloroform–methanol (2:1) and methylated with diazomethane in ethyl ether at room temperature for 15 min.

Results and Discussion

It is well known that lipid peroxides generated from PUFA play an important role in the formation of fluorescent lipofuscin pigments in aged animal tissues.[1,2] The PUFA moieties existing in biological tissues are mainly esterified in phospholipids, triglycerides and cholesterol esters, and are converted to esterified lipid peroxides without breakdown into low-molecular-weight compounds under various oxidative conditions.[7] As a model system of fluorescence formation, each HPO of ML, PLPC, TL and CL was separately incubated with 1-AP in methanol solution at 37 °C for 20 h after decomposition by heme. ML-HPO produced fluorescent substances exhibiting fluorescence spectra with excitation and emission maxima at 348 and 418 nm, respectively (Fig. 1a). PLPC- and TL-HPO also produced fluorescent substances with similar fluorescence spectra (Fig. 1b, c), as demonstrated in a previous study.[8] Similarly, CL-HPO also revealed fluorescence formation with the same fluorescence spectra as those described above through reaction with 1-AP after decomposition by heme (Fig. 1d). These results indicate that all the esterified linoleic acid-HPO in ML, PLPC, TL and CL produce fluorescent substances exhibiting similar fluorescence spectra with excitation and emission maxima at around 350 and 420 nm, respectively.

The fluorescent substances formed from ML-HPO with 1-AP in methanol solution after decomposition by heme exhibit two major fluorescence peaks at retention times of 5.0 min (FS-I) and 10.5 min (FS-II) on reverse-phase HPLC (Fig. 2a), while the fluorescent substances formed from PLPC-HPO with 1-AP elute a major fluorescence peak (FS-III_p) at a retention time of 16.0 min in addition to FS-I as described previously[9] (Fig. 2b). When the fluorescent substances formed from TL- and CL-HPO with 1-AP were subjected to reverse-phase HPLC, each major fluorescent substance was eluted at retention times of 28.5 min (FS-III_t) and 27.5 min (FS-III_c), respectively, in the present experiments (Fig. 2c, d). However, no FS-II peak was detected in these samples.

As found in our previous study,[9] the major fluorescent substance (FS-III_p) formed from PLPC-HPO remains attached to the glycerol backbone in PLPC and is hydrolyzed with phospholipase A_2. We therefore incubated each fluorescent substance prepared from TL- and CL-HPO with the respective hydrolytic enzymes, lipase and cholesterol esterase, and the hydrolyzates were methylated with diazomethane and subjected to the same HPLC. As shown in Fig. 2, the major fluorescent peak (FS-III_t) formed from TL-HPO with 1-AP disappeared after hydrolysis with lipase, and the same FS-II peak as that derived from ML-HPO appeared on HPLC (Fig. 2e). Similarly, FS-III_c formed from CL-HPO with 1-AP was also hydrolyzed with cholesterol esterase and produced FS-II with almost the same peak height on HPLC (Fig. 2f). All FS-III analogues collected by HPLC exhibited the same fluorescence spectra with excitation and emission maxima at around 350 and 420 nm, respectively. Moreover, FS-II produced from each FS-III by enzymatic hydrolysis showed the same fluorescence spectra without any changes in fluorescence intensity. Esterified fluorescent substance was formed from oxidized PLPC emulsion through reaction with 1-AP in 10 mM Tris–HCl buffer (pH 7.4) as a model system *in vivo*, and this fluorescent substance was also hydrolyzed with phospholipase A_2 (data not shown). TLC analysis of the native fluorescent substances formed from each HPO revealed one major fluorescent spot, and conversion to the respective FS-II spots occurred after enzymatic hydrolysis and methylation (data not shown). These results support the notion that esterified PUFA peroxides in phospholipids, triglyc-

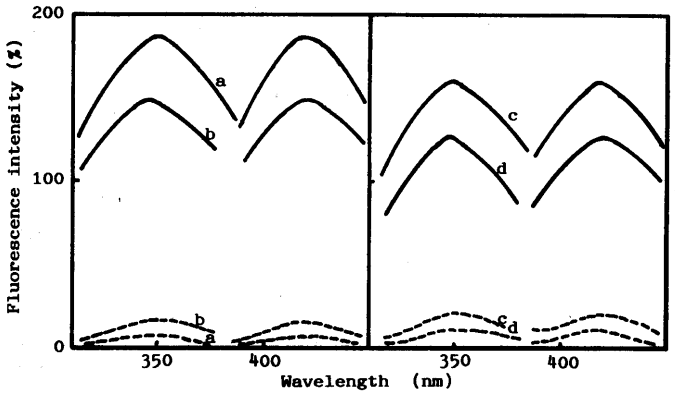

Fig. 1. Fluorescence Spectra of Fluorescent Substances Formed from HPO of ML, PLPC, TL and CL with 1-AP

Each HPO of ML (a), PLPC (b), TL (c) and CL (d) was incubated with 1-AP at 37 °C for 20 h after preincubation with (——) or without (– – – –) heme.

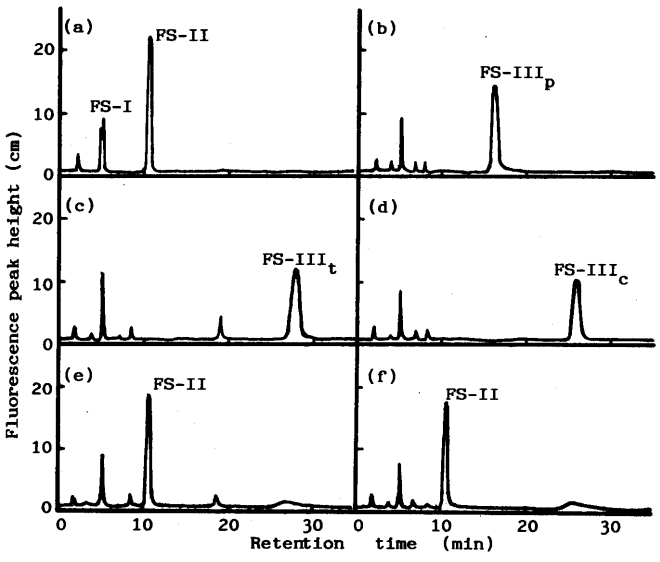

Fig. 2. HPLC of Fluorescent Substances Formed from HPO of ML, PLPC, TL and CL with 1-AP

Each fluorescent substance formed from HPO of ML (a), PLPC (b), TL (c) and CL (d) with 1-AP after preincubation with heme was subjected to reverse-phase HPLC. The fluorescent sample formed from each HPO of TL (e) or CL (f) with 1-AP was hydrolyzed with lipase or cholesterol esterase, and the hydrolyzates were methylated with diazomethane and subjected to HPLC.

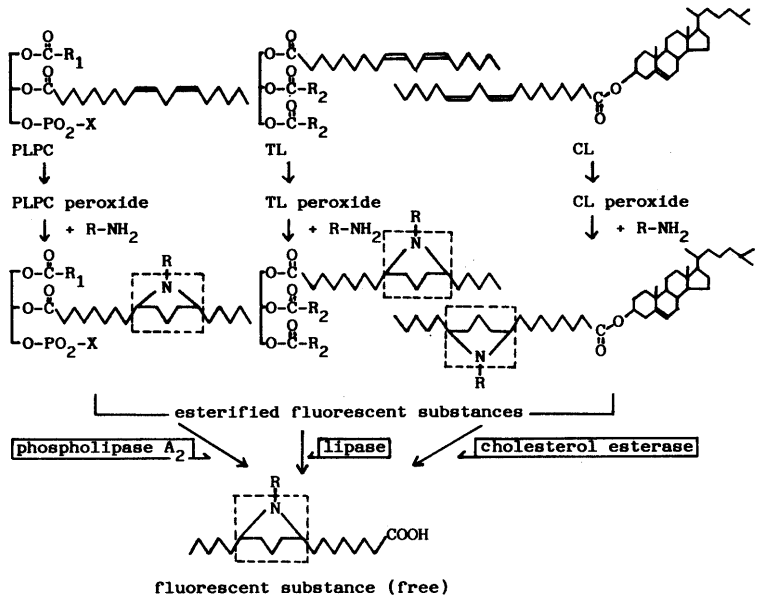

Fig. 3. Proposed Mechanism for the Formation of Esterified Fluorescent Substances from HPO of PLPC, TL and CL with Amino Compounds

The chromophobic structure enclosed with the dotted line is at present obscure. R_1, palmitic acid; R_2, linoleic acid; X, choline.

erides or cholesterol esters can produce esterified fluorescent substances without being released from their backbones, and that they are converted to similar fluorescent substances (FS-II) on HPLC despite the differences in mode of ester-linkage (Fig. 3).

We reported previously that both 9- and 13-HPO of ML produce the same fluorescent substances through reaction with amino compound after decomposition by heme.[6] Moreover, from mass spectral analysis, the FS-II formed from ML-HPO with 1-AP had an empirical formula of $C_{24}H_{41}NO_4$, with a molecular weight of 407. Other characteristic fragmentation ions (M^+-31, M^+-57, M^+-71, M^+-143 and M^+-157) showed the presence of a pentyl group and a methyl octanoate in the FS-II. This result suggests that a direct precursor available for the formation of FS-II may be a 19-carbon-length compound having a methyl group originating from ML, and that the formation of FS-II may occur in the region of the 5 carbon atoms between positions C9 and C13 in ML. The fluorescent chromophobic system remains to be clarified and is now under investigation. The above findings suggest that all 9- and 13-positional isomers of esterified linoleic acid-HPO in phospholipids, triglycerides and cholesterol ester may produce an esterified fluorescent substance having the same fluorescence chromophobic system originating from linoleic acid through reaction with amino compounds. Fukuzawa *et al.* reported that 12-keto oleic acid produced a fluorescent substance having a characteristic chromophobic system (NC=CC=O) through reaction with amino compounds without breakdown into low-molecular-weight compounds.[11] Shimasaki *et al.* also found that oxidized liposomes containing phosphatidylcholine and phosphatidylethanolamine produced fluorescent substances in liposomal membranes without being released into the medium fraction.[12]

Phosphatidylcholine-HPO and cholesterol-HPO have recently been detected in human serum by HPLC.[13,14] Moreover, phospholipids, triglycerides and cholesterol esters enriched in PUFA are contained to an appreciable extent in serum lipoproteins. Hata *et al.* reported that lipid peroxides in plasma lipoproteins of hypercholesterolemic rabbits were increased in the very-low and low-density lipoprotein fractions.[15] These peroxidized PUFA originating from phospholipids, triglycerides or cholesterol esters in serum lipoproteins may be related to fluorescence formation through reaction with amino compounds. Esterbauer *et al.* found that oxidation of low-density lipoprotein caused the formation of fluorescent substances exhibiting excitation and emission maxima at 350—370 nm and 430—450 nm, respectively.[16] Moreover, Shimasaki *et al.* reported the formation of fluorescent substances in peroxidized egg yolk low-density lipoprotein.[17] These results indicate that the formation of esterified fluorescent substances from reaction with these esterified PUFA peroxides in phospholipids, triglycerides or cholesterol esters and amino compounds may bring about the accumulation of fluorescent substances *in vivo* (Fig. 3). Moreover, the respective hydrolysis of each esterified fluorescent substance formed from PLPC-, TL- and CL-HPO by phospholipase A_2, lipase and cholesterol esterase suggests that these enzymes might play an important role in the elimination of fluorescent substances from biological tissues (Fig. 3). It is well known that phospholipase A_2 specifically hydrolyzes intact and peroxidized PUFA moieties attached at the 2-glycerol position in phospholipids.[18] Van Kuijk *et al.* have reported that the elimination of peroxidized PUFA from membrane phospholipids plays a role in the protection of membranes from peroxidation damage.[19] The elimination of peroxidized PUFA from membrane phospholipids with phospholipase A_2 also inhibits the formation of fluorescent substances through reaction with peroxidized PUFA and amino compounds in biomembranes.

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