Fluorescence Formation from Hydroperoxide of Phosphatidylcholine with Amino Compound

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The hydroperoxides of methyl linoleate, 1-palmitoyl-2-linoleoyl-phosphatidylcholine and trilinolein each produced similar fluorescent substances through reaction with amino compounds after decomposition by heme methyl ester. Fluorescent substances formed from methyl linoleate with 1-aminopentane revealed characteristic fluorescence peaks on HPLC, while those obtained from 1-palmitoyl-2-linoleoyl-phosphatidylcholine and trilinolein were not eluted under the same conditions. However, when both of these fluorescent substances were transesterified to methyl ester, the same fluorescence peaks were observed. This result suggests that fluorescent substances formed from oxidized membrane lipids with amino compounds remain attached to phospholipids without being released from their glycerol backbone.
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Polyunsaturated fatty acids (PUFA) initially are oxidized to produce hydroperoxides (HPO) by both enzymic and nonenzymic oxidations and then the HPO produced are degraded further into secondary oxidation products (SP), including many functional aldehydes or malondialdehyde, during the oxidation process (1,2). These lipid peroxides and/or their secondary decomposition products are known to react with various amino compounds, bringing about many forms of biological damage (3). Fluorescent lipofuscin pigments associated with aging in animal tissues also have been assumed to be derived from the reaction of lipid peroxides with amino compounds (4). Fujimoto et al. recently reported that the degradation of HPO prepared from methyl linoleate (ML) in the presence of metals, hemin or ascorbic acid is involved closely in fluorescence formation through reaction with DNA (5). They also suggested that the important structural feature associated with the fluorescence formation appears to be conjugated diene HPO and not unconjugated diene isomers produced during photosensitized oxidation. Therefore, HPO derived from PUFA substituted at the 2-glycerol position of phospholipids in biological membranes (6,7) may play an important role in the formation of fluorescent substances under physiological conditions.

We recently have demonstrated that SP prepared from autoxidized methyl linolenate and ML are involved in the formation of fluorescent substances through reaction with amino compounds (8,9). The high-resolution mass spectrum of the fluorescent substance (FS-II) formed from the SP with 1-aminopentane (1-AP) as a model system showed the empirical formula to be $C_{24}H_{41}NO_4$ with a molecular weight of 407 and the presence of a methyl ester group originating from ML in the structure of the fluorescent substance (Iio, unpublished data). These results suggest that the precursor available for the fluorescence formation may be a 19-carbon length compound having a methyl ester group originating from ML without breakdown into low molecular weight compounds. Fukuzawa et al. also recently have reported that a new type of fluorescent substance was produced during oxidation in the presence of amino compounds through reaction of 12-keto-oleic acid produced from oleic acid without breakdown into low molecular weight compounds (10). In the present study, we demonstrated the formation of fluorescent substances through reaction of HPO prepared from ML, 1-palmitoyl-2-linoleoyl-phosphatidylcholine (PLPC) and trilinolein (TL) with 1-AP after decomposition by heme methyl ester (HM) as a model system.

MATERIALS AND METHODS

Materials. Linoleic acid, PLPC, TL, hemin (type I) and lipoxygenase (type I) were purchased from Sigma Chemical Co. (St. Louis, MO). 1-AP and 5% methanolic *m*-trifluoromethylpheny-trimethyl ammonium hydroxide were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo). High performance liquid chromatography (HPLC) grade solvents from Kanto Kagaku Co., Ltd. (Tokyo) were used for HPLC separations throughout all experiments. HM was prepared by the method of Ortiz de Montellano et al. (11).

Preparation of HPO from ML, PLPC and TL. Two mg of PLPC or TL suspended in 1 ml of 100 mM deoxycholate solution was incubated with 9 ml of 0.1 M borate buffer (pH 9.0) containing 2 mg of lipoxygenase at 30 C for 30 min (12). The products were extracted with chloroform/methanol (2:1), and each HPO was purified by HPLC or thin layer chromatography (TLC). The purification of HPO from oxidized PLPC was carried out on a μ-Bondapak FAA (3.9 × 300 mm, Waters Associates, Milford, MA), using methanol/water (9:1) at a flow rate of 1 ml/min (13). HPO of TL was subjected to TLC (kieselgel 60, Merck, Darmstadt, FRG) developed with petroleum ether/ethyl ether/28% ammonia (55:45:2) (14). 13-Hydroperoxylinoleic acid was prepared from linoleic acid using soybean lipoxygenase in 0.1 M borate buffer (pH 9.0) (15). The products were methylated with diazomethane and purified by TLC using hexane/ethyl ether/acetic acid (60:40:1) as a solvent system. SP of ML were prepared from autoxidized ML by silica gel column chromatography according to the method of Terao and Matsushita (16). Each purified HPO was estimated using molar absorbance values of 24,500 at 233 nm (17).

Formation of fluorescent substances. Each HPO (100 nmol) prepared from ML, PLPC or TL was preincubated with or without HM (1 nmol) in 2 ml of methanol at 37 C for 45 min, and then 1 ml of 1-AP (1 μmol) in methanol solution was added to the preincubation mixtures. SP also were incubated with 1-AP in 3 ml of methanol. Fluorescence developed at 37 C during 20 hr. Fluorescence spectra were measured with a Hitachi

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Abbreviations: HM, heme methyl ester; HPLC, high performance liquid chromatography; HPO, hydroperoxides; ML, methyl linoleate; PLPC, 1-palmitoyl-2-linoleoyl-phosphatidylcholine; PUFA, polyunsaturated fatty acids; SP, secondary oxidation products; TL, trilinolein; TLC, thin layer chromatography.

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). HPLC was performed on a μ-Bondasphere phenyl column (3.9 × 150 mm, Waters) using acetonitrile/water (50:50) as the mobile phase at a flow rate of 0.7 ml/min. Fluorescence peaks were monitored with a Hitachi 650-10LC fluorescence spectrophotometer with excitation and emission maxima at 350 nm and 420 nm, respectively. All of the fluorescent substances also were subjected to TLC and developed with chloroform/methanol (9:1).

Transesterification of fluorescent material. To 0.1 ml of fluorescent material, 0.85 ml of methanol and 0.05 ml of 5% methanolic *m*-trifluoromethylphenyltrimethyl ammonium hydroxide were added, and this mixture was allowed to stand at 20 C (18). Transesterification usually was completed within 45 min. The mixture was evaporated under a nitrogen stream and redissolved in 1 ml of chloroform. This chloroform solution was applied to a Sep-pak Si cartridge (Waters), and the cartridge was washed out with 8 ml of chloroform to remove the transesterification reagent. The fluorescent substance was eluted with 4 ml of chloroform/methanol (9:1) and subjected to reversed-phase HPLC under the same conditions.

RESULTS AND DISCUSSION

When purified HPO prepared from ML, PLPC and TL were incubated separately with 1-AP in methanol solution at 37 C for 20 hr after decomposition by HM, marked formation of fluorescent substances was observed. These fluorescent substances showed the same fluorescence spectra with excitation and emission maxima at 340–350 nm and 410–420 nm, respectively, consistent with that observed for SP prepared from autoxidized ML (Fig. 1). None of the HPO preincubated without HM produced any fluorescent substances. This result suggests that HPO from the unsaturated esterified fatty acids in PLPC and TL are related equally to the formation of fluorescent substances through reaction with amino compounds in the presence of HM.

However, HPLC analysis of each fluorescent product showed independent elution profiles, as shown in Figure 2A. The fluorescent substances formed from HPO of ML with 1-AP after decomposition by HM revealed two major fluorescence peaks at retention times of 5.0 min (FS-I) and 10.5 min (FS-II), respectively. On the other

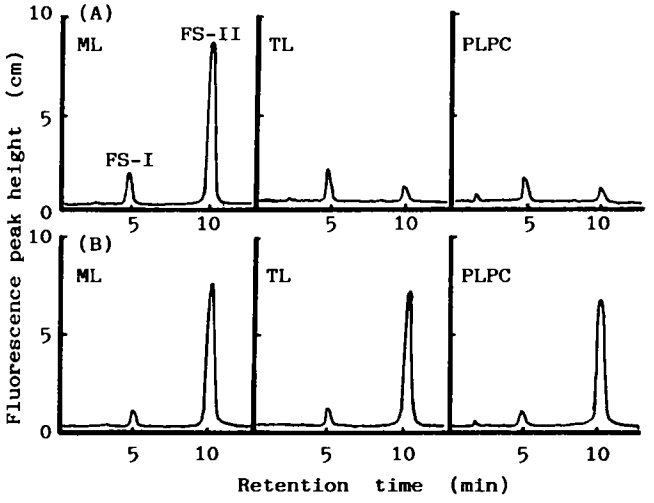

FIG. 2. HPLC of fluorescent substances formed from HPO of ML, PLPC and TL with 1-AP. HPLC was performed on a μ-Bondasphere phenyl column, and elution was carried out with acetonitrile/water (50:50) at a flow rate of 0.7 ml/min. Aliquots of the fluorescent substances were subjected to HPLC before (A) and after (B) transesterification using the procedures described in Materials and Methods.

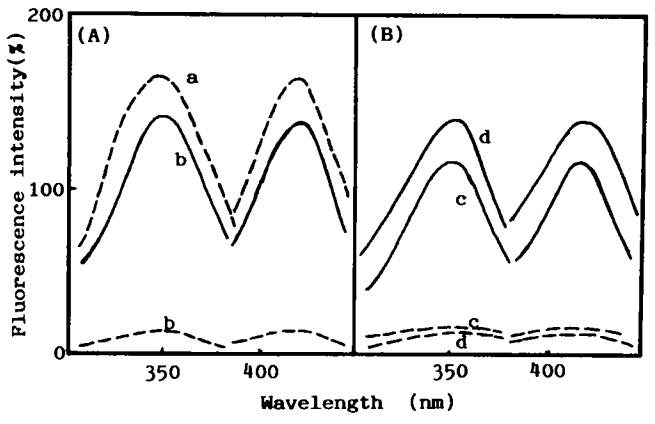

FIG. 1. Fluorescence spectra of fluorescent substances formed from SP or HPO of ML, PLPC and TL with 1-AP. HPO of ML (A-b), PLPC (B-c) and TL (B-d) were incubated with 1-AP at 37 C for 20 hr after preincubation with (——) or without (···) HM. SP prepared from autoxidized ML by silica gel column chromatography also was incubated with 1-AP at 37 C for 20 hr (A-a).

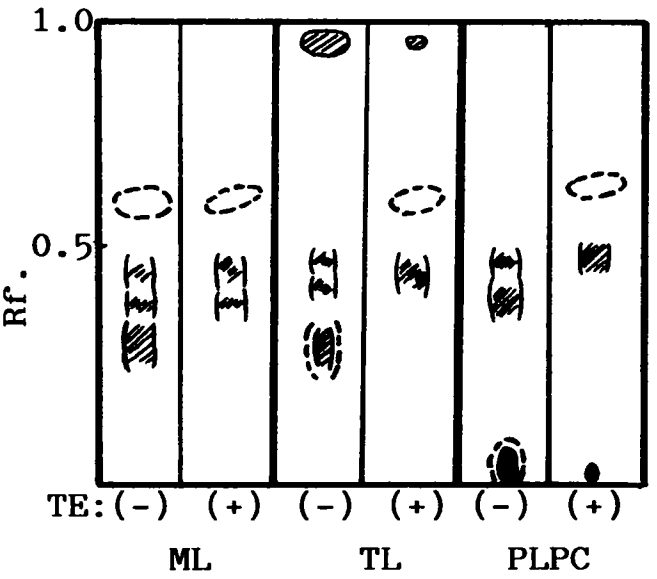

FIG. 3. TLC of flurescent substances produced from HPO of ML, PLPC and TL with 1-AP. Aliquots of fluorescent substances were subjected to TLC before (−) and after (+) transesterification (TE) and developed with chloroform/methanol (9:1). Fluorescent spots (broken circle) were detected by excitation at around 360 nm, while other spots (hatched circle) were detected using iodine vapor.

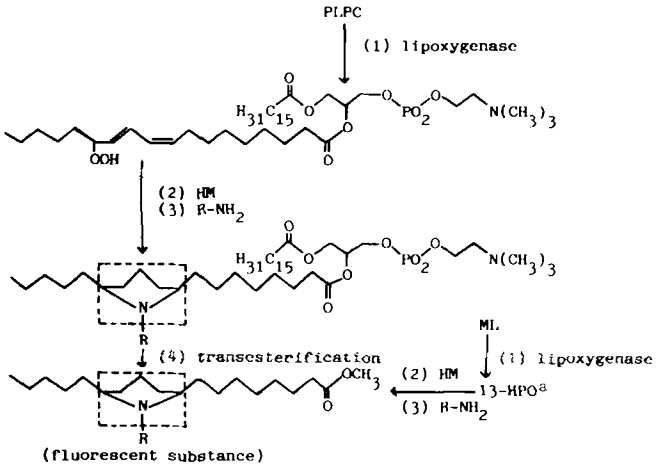

FIG. 4. Proposed mechanism for formation of fluorescent substances formed from PLPC with amino compound. The procedures (1-4) for fluorescence formation are described in Materials and Methods. The chromophobic structure enclosed with a dotted line is at present obscure. 13-HPO[a], 13-hydroperoxy methyl linoleate.

hand, the fluorescent substances derived from HPO of PLPC and TL with 1-AP showed only small fluorescence peaks at these retention times, even though samples with the same fluorescence intensities as that of ML were used for HPLC analysis and the major fluorescence peak was eluted with methanol (data not shown).

Therefore, these fluorescent substances produced from HPO of ML, PLPC and TL were transesterified to methyl esters in methanol solution and subjected to HPLC under the same conditions as those described above. Both fluorescent substances produced from HPO of PLPC and TL also were eluted at similar retention times of 10.5 min as that of FS-II obtained from HPO of ML (Fig. 2B). However, a negligible amount of FS-I was detected on HPLC after transesterification because FS-I was unstable.

TLC analysis of both the fluorescent substances produced from the HPO of PLPC and TL after transesterification to methyl ester revealed one major fluorescence spot (Rf 0.60) corresponding to FS-II formed from HPO of ML with 1-AP, while the two native fluorescent substances present before transesterification showed fluorescence spots distinct from that of FS-II on TLC (Fig. 3). The appearance of this fluorescent substance (FS-II) from the two fluorescent substances after transesterification strongly supports the possibility that HPO produced from unsaturated esterified fatty acids in phospholipids or triglycerides are involved in the fluorescence formation without being released from their glycerol backbone.

It is well known that phosphatidylcholine and phosphatidylethanolamine are the most common types of membrane lipids and that linoleic acid and arachidonic acid are abundant PUFA constituents of these phospholipids (19,20). Therefore, HPO produced from these PUFA in phospholipids by lipoxygenase or autoxidation (6,7) seem to be a primary cause of accumulation of fluorescent substances in biomembranes under physiological conditions. Nielsen reported that peroxidized phospholipid (cardiolipin) produced the fluorescent substance through reaction with albumin (21). Moreover, Shimasaki et al. have reported that the fluorescent chromolipids formed during lipid peroxidation of liposomes by ferrous ions and ascorbic acid remain in liposomal membranes (22). These reports, in addition to the results here, suggest that the hydrolytic release of unsaturated esterified fatty acids that usually are substituted at the 2-glycerol position in phospholipids is not essential for the fluorescence formation occurring in biomembranes. We recently demonstrated that both 9-hydroperoxy ML and 13-hydroperoxy ML, which are the major conjugated diene HPO produced during autoxidation of ML, formed the same fluorescent substances through reaction with 1-AP after preincubation with HM (23).

Based on the above results and the structural information mentioned above, we propose a possible mechanism for the fluorescence formation occurring during oxidation of unsaturated phospholipids in biomembranes using 13-hydroperoxides of PLPC and ML as a model compound, as shown in Figure 4. It seems likely that the precursor available for fluorescence formation keeps the original 18-carbon length of linoleic acid substituted onto phospholipids, but the structure of this precursor has not been established in detail yet.

Although fluorescent chromolipids accumulating with age in animal tissues are suggested to be produced in biomembranes, our results may support the view that fluorescent substances produced from oxidized membrane phospholipids are localized in biomembranes and accumulated without release from biomembranes.

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