Development of novel fluorescent probe 3-perylene diphenylphosphine for determination of lipid hydroperoxide with fluorescent image analysis ☆

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Abstract

A novel fluorescent probe 3-perylene diphenylphosphine (3-PeDPP) was synthesized for the direct analysis of lipid hydroperoxides. The structure of 3-PeDPP was identified by the spectroscopic data, FAB-MS, ^{1}H NMR, and ^{13}C NMR. The reactivities of 3-PeDPP with lipid hydroperoxides were investigated in chloroform/MeOH homogeneous solutions and PC liposome model systems oxidized by either 2,2′-azobis(2-amidinopropane)dihydrochloride and photosensitized oxidation. The fluorescence intensity derived from 3-perylene diphenylphosphineoxide (3-PeDPPO) increased proportionally with amount of hydroperoxides produced in homogeneous solutions and liposome model systems. 3-PeDPP was easily incorporated into mouse myeloma SP2 cells and thin tissue section for dynamic membrane lipid peroxidation studies. Linear correlations between fluorescence intensity and amount of hydroperoxides in the cell membrane and tissue sections were obtained. The fluorescence intensity from 2-dimensional image analysis was also well correlated with lipid hydroperoxide level in these models. Thus, the novel probe 3-PeDPP is useful for the direct determination of lipid hydroperoxides in biological materials.

Keywords: 3-Perylene diphenylphosphine; Fluorescent probe; Lipid hydroperoxide

Oxidative stress briefly denotes the imbalance between the concentrations of reactive oxygen species (ROS) and the antioxidative defense mechanisms [1]. Lipid peroxidation is one of the commonly occurring cellular processes and is involved in cell adhesion and proliferation, inflammatory responses, reproduction, aging, and death. The progress of lipid peroxidation has been shown to be associated with the disturbance of fine structure and the function loss of the biological membranes [2]. The lipid peroxidation is preceded by enzymatic or non-enzymatic pathways and latter pathways depend on temperature, light, and free radical generators. The initial products of lipid peroxidation are lipid hydroperoxides, which are used in assessing the extent of oxidative stress. Membrane phospholipids and triacylglycerides containing unsaturated fatty acids are thought to be the primary sites of the lipid peroxidation process. The oxidation of membrane phospholipids has been hypothesized to cause an increase in the permeability of cell membrane leading to cell death [3].

Phosphines are well known to stabilize transition metals in low oxidation states but their versatility as ligands is also documented by their ability to coordinate to transition metals [4]. The triarylphosphine molecule having fluorophore instead of phenyl group of triphenylphosphine showed changed fluorescence spectra with oxidation of phosphorus [5] such as diphenyl-1-pyrenylphosphine (DPPP). DPPP is also used for the determination of phospholipid hydroperoxide by thin-layer chromatographic blotting [6], hydroperoxides of phosphatidylcholine,

☆ *Abbreviations:* 3-PeDPP, 3-perylene diphenylphosphine; 3-PeDPPO, 3-perylene diphenylphosphineoxide.

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phosphatidylethanolamine, and cholesteryl ester by high-performance liquid chromatography with post-column derivatization [7,8], and lipid hydroperoxide in cell membrane [9,10]. However, a disadvantage of DPPP is the fluorescence excitation and emission wavelengths inside the UV region, causing cell death and inducing lipid peroxidation by itself. The purpose of this study was to design and synthesize a novel fluorescent probe 3-perylene diphenylphosphine (3-PeDPP) having a fluorophore perylene instead of pyrenyl group and to supply a novel fluorescent probe for determining and directly monitoring lipid peroxidation in biological materials with an excitation wavelength safer for cell viability.

Materials and methods

Materials. Triphenylphosphine (99%), perylene (>99%), tetrahydrofuran anhydrous (99.9%), lithium (wire with a 3.2 mm diameter, 99.9%), copper (II) chloride (97%), 2-chloro-2-methylpropane (99%), 2,2′-azobis(2-methylpropionamidine)dihydrochloride (97%), copper (II) bromide (99.999%), rose bengal, and cumene hydroperoxide (80%) were purchased from Sigma–Aldrich Japan (Tokyo, Japan). Carbon tetrachloride was purchased from Wako Pure Chemical (Osaka, Japan). Cosmosil 75 C18-PERP was purchased from Nacalai Tesque (Kyoto, Japan). Silica gel 60 (spherical 40–50 μm) was purchased from Kanto Chemical (Tokyo, Japan). 1-Palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (16:0,18:2 PC) was purchased from Avanti (Alabaster, AL, USA). Other organic solvents and chemical reagents were of the analytical grade and used without further purification.

Preparation of 3-bromoperylene. 3-Bromoperylene was synthesized using a procedure according to the method of Nohebel [11]. A mixture of perylene (5.0 g, 0.020 mole), copper (II) bromide (5.0 g, 0.022 mole), and carbon tetrachloride (400 ml) was stirred at reflux temperature for 6 days. Afterwards, the mixture was filtered to remove the residual copper (II) bromide and the resulting solution was separated by silica gel 60 column chromatography using benzene as an eluent.

Preparation of 3-PeDPP. 3-PeDPP was synthesized according to the method of Akasaka et al. [12]. A mixture of triphenylphosphine (5.0 g, 0.019 mole), lithium (500 mg), and tetrahydrofuran (200 ml) was stirred at reflux temperature for 3 h, and the red-brown solution was obtained. 2-Chloro-2-methylpropane (1.8 g) in 20 ml of tetrahydrofuran was added and refluxed for another 10 min and then the residual lithium was filtered through glass wool column packed loosely. Afterwards, red-brown solution was mixed with 3-bromoperylene and stirred at reflux temperature for 3.5 h. The mixture was washed with chloroform (2×50 ml) and finally dried under vacuum to afford the crude 3-PeDPP.

Purification of 3-PeDPP. Crude 3-PeDPP was purified by flash chromatography on silica gel 60 (spherical 40–50 μm) and cosmosil 75 C18-PREP using hexane plus benzene (8:2, v/v) and acetonitrile as eluents, respectively. 3-PeDPP was purified with a HPLC system, equipped with an ODP-50 column (250 mm × 6.0 mm) from Asahi Chemical Industry (Tokyo, Japan). The column was equilibrated for 30 min with a mobile phase, acetonitrile plus water plus acetic acid (90:10:0.01, v/v/v), with a constant flow rate of 1.0 ml/min prior to sample injection. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectra were taken in $CDCl_3$ at 600 MHz with a JEOL A600 NMR spectrometer. Mass spectra of 3-PeDPP and their oxide were obtained by fast atom bombardment (FAB) mode with a JEOL JMS-700 mass spectrometer. Fluorescence spectra of 3-PeDPP and 3-perylene diphenylphosphineoxide (3-PeDPPO) were obtained with a spectrofluorometer RF-1500 (Shimadzu, Kyoto, Japan).

Liposome preparation. Unilamella vesicles were prepared according to the method of Chen et al. [13] by injecting ethanol solution (500 μl) which contained 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (16:0,18:2 PC) 12.5 mg into 5 ml of distilled water under stirring. The solution was further diluted with distilled water and the final concentration of PC was adjusted to 0.625 mg lipid per ml. The vesicles were analyzed for the size distribution by dynamic light scattering. Average diameter of unilamella vesicles was determined as 90–100 nm by a Coulter Sizer N4Plus (Beckman Coulter Japan, Tokyo, Japan).

Oxidation procedures of 3-PeDPP in homogeneous solution and PC liposome with an oxidant or visible light with a photosensitizer. The reactivity of 3-PeDPP with cumene hydroperoxide was analyzed at 37 °C in chloroform/methanol (1:1, v/v) solution containing 15 μM of 3-PeDPP. Fluorescence intensity with the 3-PeDPPO generation was continuously monitored during 2400 s. In liposome model systems, lipid peroxidation was induced by the addition of an azobis radical initiator, AAPH. For photosensitized oxidation, PC liposome solutions were irradiated in the presence of rose bengal (20 μM) in a cold room at 5–6 °C through a 3-cm layer of water to filter out infrared radiation, using a 100 W tungsten light source (4500 lux). At appropriate intervals, aliquots of the PC liposome solutions were added with 3-PeDPP in methanol solutions at a final concentration of 15 μM and final volume 3.0 ml. The fluorescence intensity of 3-PeDPPO was measured after appropriate incubation time.

Preparation of mouse myeloma SP2. Mouse myeloma SP2/O-AG14 (SP2) cells (1.5×10^6 cells/ml) were cultured in 6-well tissue culture plates (Costar, USA) with a GIT medium (Wako, Osaka, Japan) at 37 °C in a humidified 5% CO_2 atmosphere. SP2 cells were collected and incubated with AAPH at a final concentration of 1 mM at 37 °C between 0 and 3 h. At the end of incubation period, SP2 cells were washed twice with PBS and labeled with 3-PeDPP in PBS at a 15 μM of final concentration at 37 °C for 15 min in the dark. After washing with PBS, 3-PeDPP-labeled cells were observed by fluorescence microscopy and spectrofluorometer RF-1500 (Shimadzu, Kyoto, Japan) with excitation and emission wavelengths at 440 and 470 nm, respectively.

Animals. Female obese diabetic db/db mice (BKS.Cg-+*Lepr^{db}*/+*Lepr^{db}*/Jcl) and female c57BL/6Jcl wild-type mice (8 weeks of age) were obtained from Clear Japan (Tokyo, Japan). The mice were kept ($n = 3$ per cage) in the room with 50% relative humidity and a 12/12-h light/dark cycle at 20–22 °C. Experimental mice were fed with water and a commercial non-purified diet type MF (Oriental Yeast, Tokyo, Japan) for 2 months until they were 16 weeks old. The body weights of the obese and diabetic and the non-diabetic mice were 50–60 and 25–30 g in 16 weeks old, respectively. The obese diabetic status was confirmed by the blood glucose level with using Glucose CII test kit (Wako Chemicals (Osaka, Japan)). After general anesthesia with diethyl ether, the main artery was perfused with PBS, and then the liver and heart were excised out. One-millimeter sections ($n = 3$) were taken from different lobes and stained with PBS plus DMSO solution (9:1, v/v) containing 3-PeDPP at the 3.43 μM concentration for 15 min at room temperature. The remaining parts of the tissues were stored at −85 °C for the following determination of lipid hydroperoxide levels.

Image analysis. Fluorescence microscopy and digital image acquisition were carried out using an Olympus IX70 inverted microscope (Olympus, Tokyo, Japan), UApo 40 lens; U-MNBV cube filter (420–440 nm excitation, 475 nm emission) equipped with an image intensified CCD camera (C2400-89, Hamamatsu Photonics, Hamamatsu, Japan). Data were analyzed by an ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Measurement of lipid hydroperoxide in cells and tissue by xylenol-orange method. The lipids were extracted from SP2 cells (1.5×10^6 cells) in 2:1 chloroform/methanol according to Folch et al. [14]. The lipid hydroperoxide levels for cells and tissue were determined by xylenol-orange method according to Gebicki et al. [15] and Hermes-Lima et al. [16], respectively. Levels of lipid hydroperoxide were expressed as CuOOH equivalent.

Results

Preparation of 3-PeDPP

The synthesis of 3-PeDPP was performed according to the reactions outlined in Fig. 1; perylene was converted

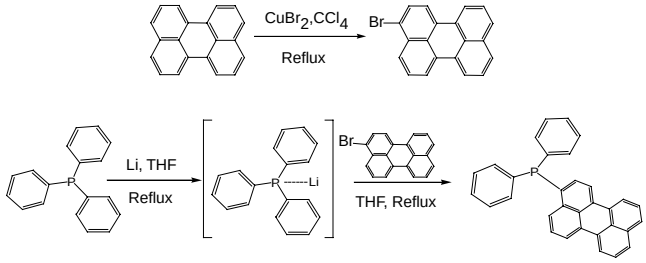

Fig. 1. Synthetic fluorometric probe 3-perylene diphenylphosphine (3-PeDPP).

to the 3-bromoperylene on the reaction with copper (II) bromide in a satisfactory yield of 65%. After drying a suspension of 3-bromoperylene in vacuum, the brownish crystalline was obtained. The reaction of triphenylphosphine with lithium in tetrahydrofuran produced [Li-PPh_3]. Finally, through an aryl exchange reaction with the 3-bromoperylene in [Li-PPh_3], the molecules of 3-PeDPP were obtained.

The crude product of 3-PeDPP was isolated through silica flash chromatography and further purified by ODS column chromatography. All fractions containing 3-PeDPP showed the characteristic yellowish color in benzene. The yield of 3-PeDPP was 51% when 3-bromoperylene was allowed to react with triphenylphosphine for 3 h.

The structure of 3-PeDPP was characterized by mass spectrometry and interpretation of 2D NMR data including COSY, HSQC, HMBC, and ^{1}H-^{31}P-HMBC spectra. The assignments for the ^{1}H and ^{13}C signals were as follows: ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.97 (dd, $J = 4.6$, 7.7 Hz, H-2), 7.42 (t, $J = 7.9$ Hz, H-5), 7.44 (t, $J = 7.9$ Hz, H-11), 7.46 (t, $J = 7.9$ Hz, H-8), 7.66 (d, $J = 8.1$ Hz, H-9), 7.67 (d, $J = 8.1$ Hz, H-10), 8.03 (2H, d, $J = 7.7$ Hz, H-1 and H-12), 8.17 (d, $J = 7.3$ Hz, H-7), 8.19 (d, $J = 7.7$ Hz, H-6), 8.26 (dd, $J = 4.6$, 8.4 Hz, H-4). ^{13}C NMR (150 MHz, $CDCl_3$) δ 136.6 (d, $J_{C\text{-}P} = 22.5$ Hz, C-3a), 136.2 (C-3), 134.5 (C-9a), 132.5 (C-2), 132.1 (C-12b), 131.6 (C-6a), 131.2 (C-6b), 130.9 (C-12a), 128.7 (C-12c), 128.4 (C-12d), 128.2 (C-10), 127.9 (C-9), 126.8 (d, $J_{C\text{-}P} = 4.5$ Hz, C-5), 126.6 (C-8), 126.5 (C-11), 126.0 (d, $J_{C\text{-}P} = 27.0$ Hz, C-4), 120.4 (C-6, C-7, C-12), 119.8 (C-1). MS-FAB Calcd for [$C_{32}H_{21}P$]: 436.48, found 436.13 and [$C_{32}H_{21}PO$]: 452.48, found 452.13. These data illustrated the structure of 3-PeDPP and their oxide as shown in Fig. 2.

Reactions of 3-PeDPP in homogeneous solution and PC liposome model

The fluorescence emission spectra data for 3-PeDPP and their oxide are shown in Fig. 3, which clearly illustrates that the 3-PeDPP is an almost non-fluorescent compound. The fluorescence intensity increased with oxidation of the 3-PeDPP into 3-PeDPPO through the cumene hydroperoxide treatment. The excitation and emission peak wavelengths were 440 and 470 nm, respectively. The same spectra were obtained when the 3-PeDPP was oxidized by other hydroperoxide reagents such as hydrogen peroxide (data not shown).

For a better understanding of the mechanism of fluorescence expression, some preliminary experiments were done to ensure that the enhancement of fluorescence intensity was indeed due to the reaction of 3-PeDPP with hydroperoxide. Before the addition of hydroperoxide to the solution of chloroform plus methanol (1:1, v/v), the fluorescence of this homogeneous solution was minimal. With the addition of hydroperoxide, a large enhancement of fluorescence intensity of produced 3-PeDPPO was observed as shown in Fig. 4A. The fluorescence intensity measured by spectrofluorometer (ex 440 nm and em 470 nm) increased exponentially during 15 min. The fluorescence intensity of

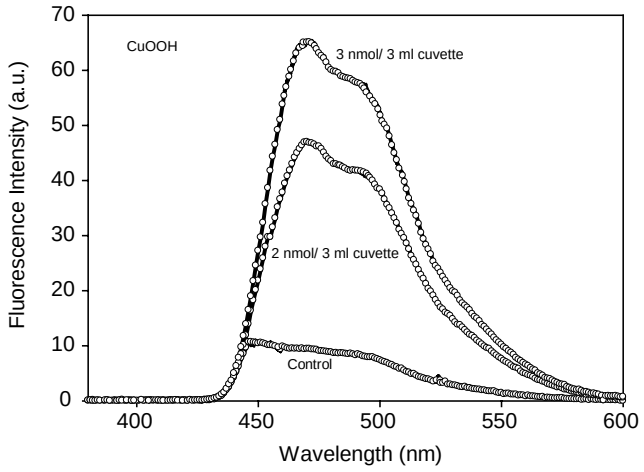

Fig. 3. Emission spectra of synthetic fluorescence probe (3-PeDPP) before (control) and after incubation with cumene hydroperoxide at the final concentration of 2 and 3 nmol per 3 ml cuvette for 40 min at 37 °C.

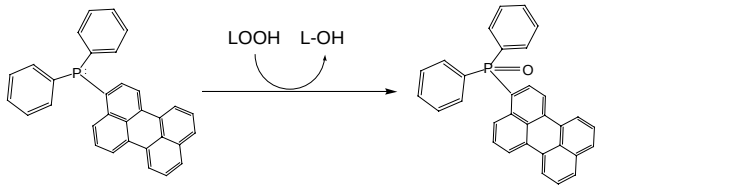

Fig. 2. Reaction of 3-PeDPP with hydroperoxide.

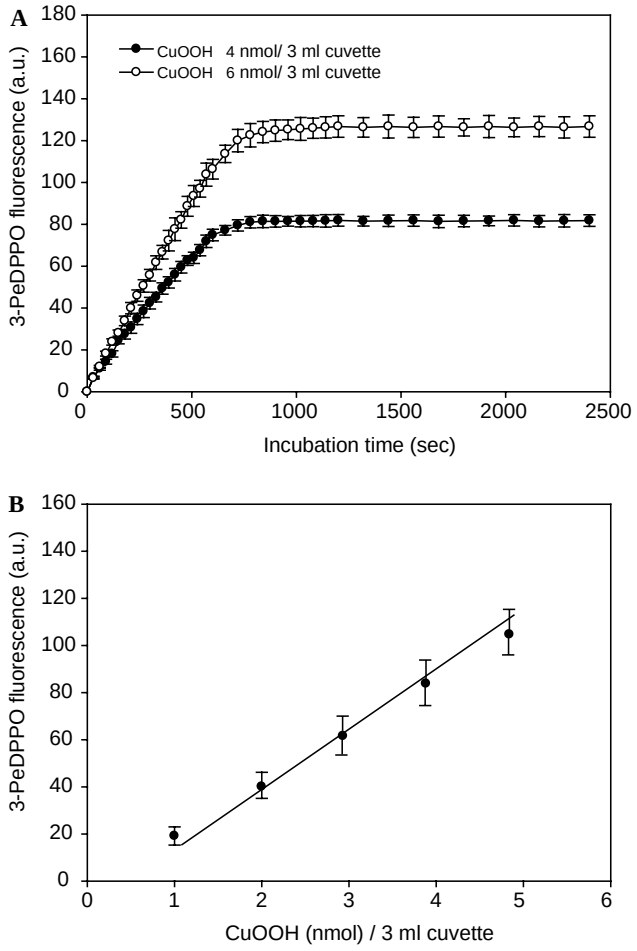

Fig. 4. Effects of oxidants on 3-PeDPP fluorescence in homogeneous solution (chloroform/methanol 1:1 v/v). Fluorescence of 3-PeDPPO was measured continuously after addition of the cumene hydroperoxide for 2400 s. (A) Time course of the 3-PeDPPO formations was measured by spectrofluorometer (ex 440 nm and em 470 nm). (B) Dose-dependencies for the 3-PeDPPO formation ($n = 3$).

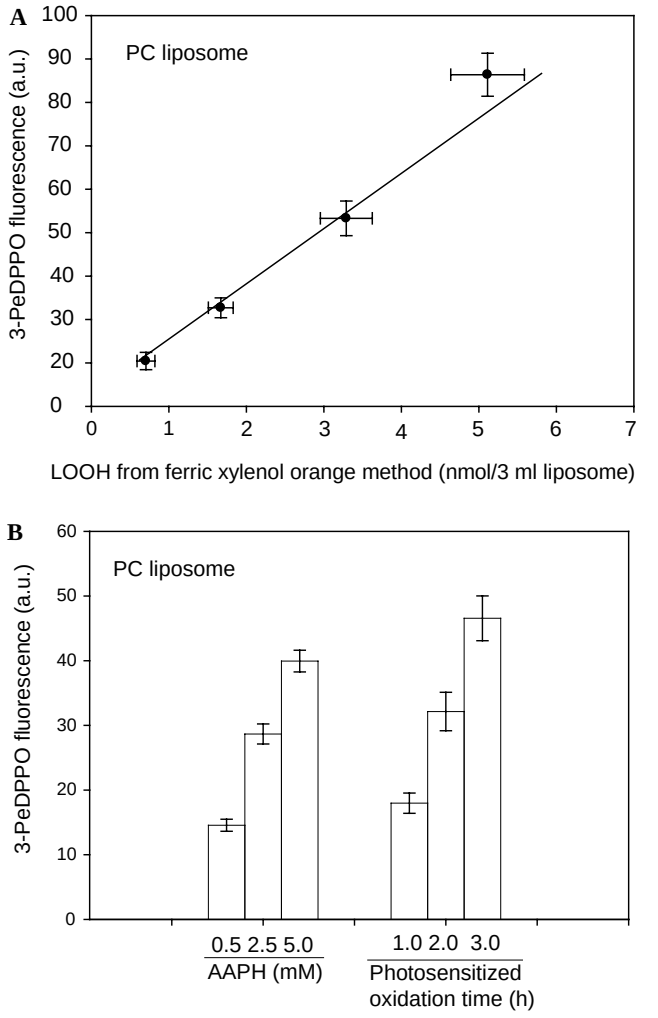

Fig. 5. (A) Relationship between lipid hydroperoxide determined by xylenol orange and fluorescent probe 3-PeDPP for phospholipid 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (16:0, 18:2 PC) liposome oxidized by AAPH (5–20 mM, 3 h at 37 °C). (B) Fluorescence intensity of 3-PeDPPO in phospholipid 16:0, 18:2 PC liposome model oxidized by AAPH or visible light in the presence of 20 μM of rose bengal. The fluorescence intensity of 3-PeDPPO was measured by spectrofluorometer (ex 440 nm and em 470 nm). All experiments were performed at 37 °C in air and the concentration of 3-PeDPPO was 15 μM. Data are expressed as mean values with SD bars ($n = 3$).

3-PeDPPO in homogeneous solution showed a significant increase with increase in the amount of cumene hydroperoxide (Fig. 4B). Furthermore, the fluorescence intensity increased proportionally with concentrations of hydroperoxide produced in homogeneous solutions. We found a negligible increase of fluorescence in the absence of hydroperoxide, demonstrating the stability of 3-PeDPP in homogeneous solution. We also confirmed that the fluorescence of 3-PeDPPO increased in the phospholipid liposome model system oxidized by AAPH or photosensitized oxidation. Fig. 5A shows the correlation between lipid hydroperoxide determination with ferric-xylenol orange complex and fluorescence intensity of 3-PeDPPO of phospholipid liposome oxidized by AAPH (5–20 mM, 3 h at 37 °C). The fluorescence intensity of 3-PeDPPO was well-correlated lipid hydroperoxide contents ($R^2 = 0.991$). Fig. 5B shows that the fluorescence intensity of 3-PeDPPO with AAPH treatment or photosensitized oxidation increased by the increasing concentration and irradiation time.

Estimation of the lipid peroxidation in cell models

The 3-PeDPP was used as a fluorescent probe for determination of lipid peroxidation of the mouse myeloma SP2 cell model system. The generating peroxyl radical AAPH was known to directly initiate lipid peroxidation in many types of membranes [17]. The lipid hydroperoxide levels in SP2 cells during incubation with AAPH at a final concentration of 1 mM gradually increased during 3 h of incubation. The AAPH-induced increase in fluorescence intensity of 3-PeDPP-labeled SP2 cells is shown in Fig. 6. The relationships between 3-PeDPPO fluorescence in spectrofluorometer or from fluorescent image analysis and the lipid hydroperoxide levels are shown in Fig. 7. There are very high correlations

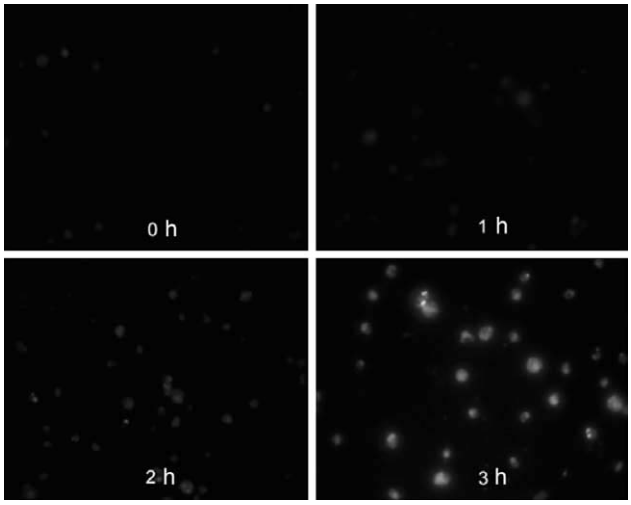

Fig. 6. Representative images of 3-PeDPP-labeled SP2 cells (1.5×10^6 cells) during incubation with AAPH (1 mM) from 0 to 3 h at 37 °C, using fluorescence microscopy equipped with an image-intensified CCD video camera.

between lipid hydroperoxide levels and 3-PeDPPO fluorescence determined by spectrofluorometer ($R^2 = 0.988$) or fluorescent 2-dimensional image analysis ($R^2 = 0.982$).

Estimation of lipid hydroperoxide in tissue slice models

3-PeDPP was also addressed to examine directly the lipid hydroperoxide production in liver and heart tissues from obese diabetic db/db mice. The blood glucose levels of control and obese diabetic mice were 167.8 and 421.8 mg/dl, respectively. After loading with the fluorescent probe, marked increases of 3-PeDPPO fluorescence were found in the liver and heart tissues of obese diabetic db/db mice (Figs. 8B and D), which reflected high levels of lipid hydroperoxide. The low fluorescence intensity in liver and heart mice of the control group with using 3-PeDPP illustrated low level of lipid hydroperoxide in the tissues (Figs. 8A and C). 3-PeDPPO fluorescence from image analysis was 33.06 ± 3.56 and 16.76 ± 2.44 (arbitrary unit) for liver and heart of control mice, respectively, but liver and heart of obese diabetic db/db mice showed 101.81 ± 12.25 and 82.22 ± 9.34 (arbitrary unit), respectively. Moreover, lipid hydroperoxide levels measured through the ferric xylenol-orange method were significantly higher in obese diabetic db/db tissues (400.10 ± 27.81 for liver and 278.44 ± 21.62 nmol/g sample for heart) than those of the control group tissues (117.62 ± 20.28 for liver and 67.17 ± 17.78 nmol/g sample for heart). These results also showed very good correlation ($R^2 = 0.982$) between values with the 3-PeDPPO 2-dimensional fluorescence image analysis and ferric-xylenol orange method in tissues (Fig. 9).

Discussion

Several pieces of evidence have suggested that the lipid peroxidation serves as a marker of cellular oxidative stress

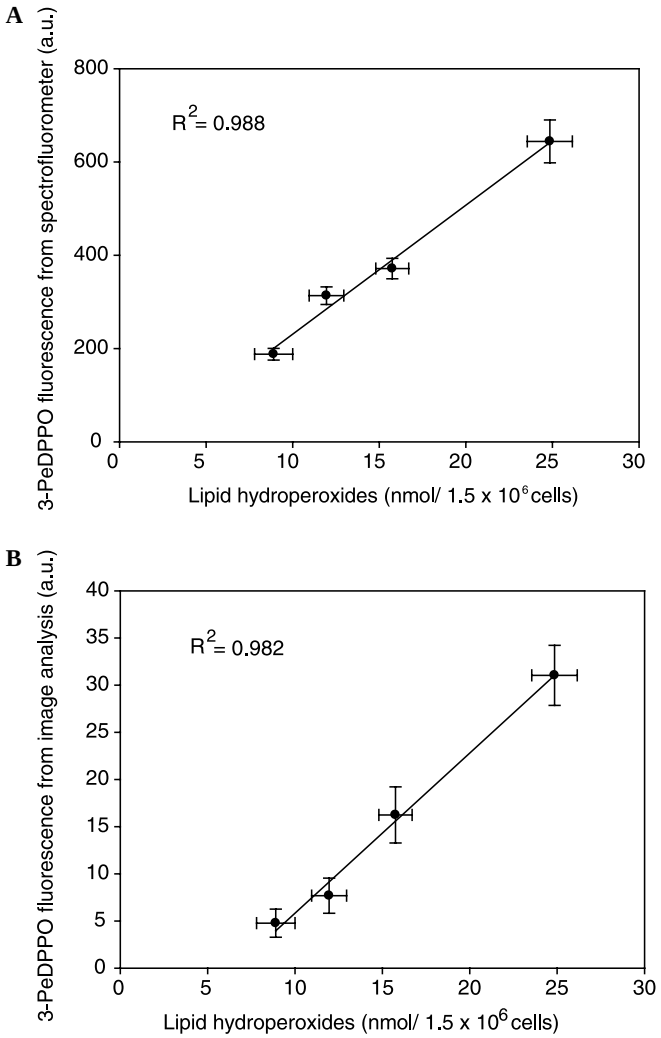

Fig. 7. Relationships between 3-PeDPPO fluorescence in labeled-SP2 cells (15 μM, 1.5×10^6 cells) measured by spectrofluorometer (A) and fluorescent 2-dimensional image analysis (B) and lipid hydroperoxide level measured by ferric xylenol orange method.

and has long been recognized to contribute to cellular oxidative damage [18]. The fluorometric method appears to be a better indicator of oxidative stress since the increasing fluorescence intensity from the reaction of triarylphosphines with hydroperoxide seems to be a general phenomenon of triarylphosphines having fluorophore instead of phenyl group of triphenylphosphine (TPP) [5]. And it can be applied for quantitative analysis of lipid peroxidation [12]. In this study, we decided to use perylene instead of the phenyl group in TPP molecule because its excitation wavelength (440 nm) is above UVA excitation wavelength (320–400 nm), which is safe for cell and biological materials [19]. The 3-PeDPPO, which was from the reaction between 3-PeDPP and hydroperoxide (Fig. 2), showed the strong fluorescence intensity with excitation and emission wavelengths at 440 and 470 nm (Fig. 3), because this reactivity was accelerated by decreases of the steric hindrance around the phosphorus atom and increase of electron density on the phosphorus atom [5].

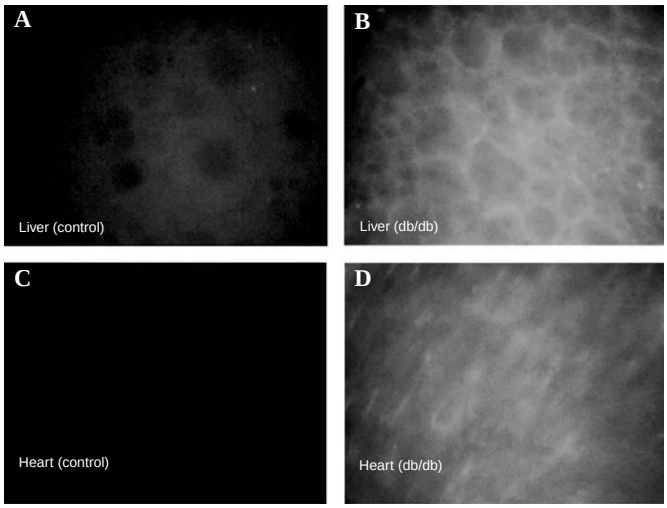

Fig. 8. Representative fluorescent images of mice liver and heart thin section stained with PBS containing 3-PeDPP at the concentration of 3.43 μM for 15 min. (A,B) Images of liver tissue of 16 weeks c57BL/6Jcl (control mice, A), 16 weeks BSK.Cg-+*Lepr*^{db}/+*Lepr*^{db}/Jcl (obese diabetic *db*/*db* mice, B). (C,D) Images of heart tissue of 16 weeks c57BL/6Jcl (control mice, C), and 16 weeks BSK.Cg-+*Lepr*^{db}/+*Lepr*^{db}/Jcl (obese diabetic *db*/*db* mice, D).

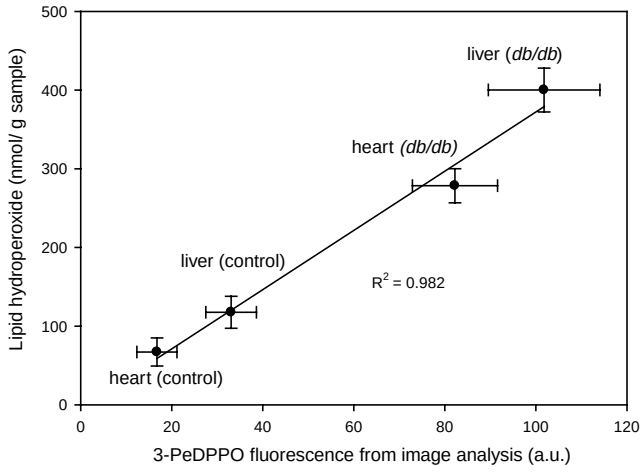

Fig. 9. Relationship between the lipid hydroperoxide levels in tissues of obese diabetic determined by 3-PeDPPO fluorescent image analysis and ferric xylenol orange method. Data are represented as means ± SD ($n = 3$).

As shown in Fig. 4A, the fluorescence intensity of 3-PeDPPO with the various reaction times was increased dramatically with a higher amount of cumene hydroperoxide, which is the same result between reaction of DPPP and hydroperoxide [20,21]. In addition, the concentration of hydroperoxide linearly correlated to 3-PeDPPO production (Fig. 4B). The application range of 3-PeDPPO fluorescence in the homogeneous solution was 0.1–60.0 nmol of CuOOH in a 3-ml cuvette with a good correlation according to our previous report [22]. On the other hand, the application range of the xylenol-orange method was 0.6–65.0 nmol of CuOOH in a 3-ml cuvette with a good correlation (data not shown). From these results, it is supposed that the 3-PeDPPO fluorescence method is more sensitive than the xylenol orange method.

Unilamellar PC liposomes were used in this study, since they mimic biological membrane systems more closely than multilamellar liposomes. In the present study, all AAPH and photosensitized oxidation induced PC liposome peroxidation. First of all, we showed the linear regression between the lipid hydroperoxide levels from xylenol-orange method and the fluorescence intensity of 3-PeDPPO ($R^2 = 0.991$, Fig. 5A). From this result the fluorescence intensity of 3-PeDPPO can be used as the index for quantitative analysis of lipid hydroperoxide in non-homogeneous solution. This study showed the linear correlations between increases in fluorescence intensity of 3-PeDPPO and concentration of AAPH (Fig. 5B). This finding was well consistent with the report by Kubo et al. [23] that the reaction rate depends only on the concentration of AAPH with regard to the acyl chain region of PC molecules, containing unsaturated chains, such as linoleic acid, which would be expected that phospholipid free radical generation by AAPH occurs on the surface of liposome. Fig. 5B also shows that the longer photosensitized oxidation time causes the stronger fluorescence intensity of 3-PeDPPO from efficient photosensitizer or photoexcitation under aerobic condition, resulting in that generation of singlet oxygen leads to formation of hydroperoxides [24].

Cells in general undergo lipid peroxidation in two general ways by generating reactive oxygen species or by the reaction of specific enzyme, such as lipoxygenase, on lipids present in cell membrane. Our studies were designed to quantify the lipid hydroperoxide level in SP2 cell membrane by 3-PeDPPO fluorescence. The present results showed the enhanced lipid hydroperoxide production in SP2 cells during incubation with AAPH at 37 °C. As expected, the higher fluorescence intensity of 3-PeDPPO was also observed in SP2 cells membrane during a longer incubation time with AAPH at 37 °C (Fig. 6). Moreover, lipid hydroperoxide level of SP2 cells was measured by using spectrofluorometer and fluorescent 2-dimensional image analysis (Figs. 7A and B), which express the strong correlation between fluorescence intensity of 3-PeDPPO and lipid hydroperoxide level in SP2 cells. Thus, this probe can be used as an indicator of general lipid peroxidation of the cell model.

Oxidative damage has been suggested to be a contributory factor in the development and complication of diabetes [25]. Many mechanisms were suggested to contribute to the formation of reactive oxygen free radicals [26]. This section was designed to quantify the lipid hydroperoxide in liver and heart tissues of type 2 diabetic (BKS.Cg-+*Lepr*^{db}/+*Lepr*^{db}/Jcl) and control (C57BL/6Jcl) mice. The present results showed the enhanced lipid hydroperoxide production in the liver and heart of obese diabetic db/db mice. The higher fluorescence intensity of 3-PeDPPO was successfully observed in obese diabetic db/db mice liver and heart tissues in comparison with those of control mice (Figs. 8A–D). This

finding indicates that lipid hydroperoxide was higher in liver and heart tissues of the obese diabetic db/db mice than the control mice, which was well consistent with the xylenol-orange method results (Fig. 9). The results were in agreement with the previous reports that the levels of lipid hydroperoxide and oxidative stress increase in diabetes mellitus [27–29]. Moreover, lipid hydroperoxide levels of the obese diabetic db/db and control mice were measured by using fluorescent image analysis, which express the strong correlation between fluorescence intensity of 3-PeDPPO and lipid hydroperoxide levels in liver and heart tissues. Thus, 3-PeDPPO fluorescence image analysis will be useful for the spatio-temporal investigations of lipid peroxidation in biological tissues.

The present study shows that this novel method with 3-PeDPP has several advantages. The reaction of 3-PeDPP and hydroperoxide to give 3-PeDPPO is suitable for measurement of lipid hydroperoxide in homogeneous solution, non-homogeneous solution such as liposome, cell membrane, and mice tissue sections. Another advantage is that 3-PeDPP has the milder excitation wavelength about 440 nm, outside the UV region safer to cell viability, and hardly inducing lipid peroxidation by itself. It is concluded that 3-PeDPP as a powerful fluorescent probe can be applied for a real-time monitoring of lipid peroxidation in biological tissues and materials.

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