A novel fluorescent probe diphenyl-1-pyrenylphosphine to follow lipid peroxidation in cell membranes

Yuko Okimoto[a], Akira Watanabe[a], Etsuo Niki[a], Takashi Yamashita[b], Noriko Noguchi[a,*]

[a] *Research Center for Advanced Science and Technology, University of Tokyo, 4-6-1 Komaba, Meguro, Tokyo 153-8904, Japan*
[b] *Department of Chemistry and Biotechnology, Faculty of Engineering, University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-8656, Japan*

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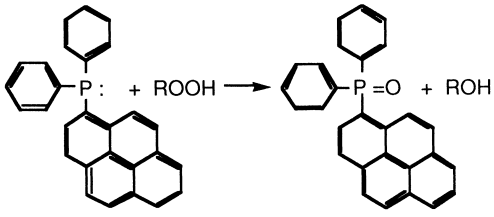
Abstract Diphenyl-1-pyrenylphosphine (DPPP) was tested whether it could be used as a fluorescent probe to monitor lipid peroxidation in cell membranes. DPPP reacted with organic hydroperoxides and hydrogen peroxide stoichiometrically to give DPPP oxide (DPPP=O). DPPP incorporated into phosphatidylcholine liposomal membranes and polymorphonuclear leukocytes (PMNs) reacted with methyl linoleate hydroperoxide rapidly but not with hydrogen peroxide nor with *tert*-butyl hydroperoxide. This novel method revealed that lipid peroxidation proceeded within membranes of PMNs stimulated with phorbol 12-myristate 13-acetate, which is known to produce several kinds of free radicals. It was found that DPPP is a suitable fluorescent probe to monitor lipid peroxidation within cell membranes specifically.

Key words: Lipid peroxidation; Polymorphonuclear leukocyte; Imaging; Diphenyl-1-pyrenylphosphine; Fluorescent probe

1. Introduction

Lipid peroxidation has been implicated in the pathogenesis of various diseases including atherosclerosis [1]. Polyunsaturated fatty acids and their esters are the major target of active oxygen and nitrogen species and their oxidation in the membranes has been accepted to play an important role in the oxidative stress in vivo. The extent of lipid peroxidation has been assessed by thiobarbituric acid reactive substances [2], lipid hydroperoxides themselves [3], and their degradation products such as malondialdehyde and 4-hydroxy-2-nonenal [4]. More recently, monoclonal antibodies raised against 4-hydroxy-2-nonenal and acrolein have also been developed and applied [5,6]. These methods have their own merits and demerits. In order to follow the oxidation taking place in cells and tissues, fluorescent dyes such as dichlorodihydrofluorescein diacetate (DCFH-DA) [7] and dihydrorhodamine (DHR) 123 [8] have been widely used. DCFH-DA is trapped within cells in the form of DCFH. DCFH and DHR 123 are not fluorescent per se but they are oxidized by H_2O_2 and other oxidants to yield fluorescent compounds, although it has been argued whether H_2O_2 reacts with DCFH directly [9,10]. Fluorescent analysis enables continuous observation of living cells and tissues with microscopy during oxidation. However, since lipid peroxidation takes place within the membranes, both DCFH and DHR 123, which are hydrophilic, are not suitable for measuring it.

Diphenyl-1-pyrenylphosphine (DPPP) is known to react stoichiometrically with hydroperoxide to give diphenyl-1-pyrenylphosphine oxide (DPPP=O) and alcohol as shown in Eq. 1 [11]. DPPP is a non-fluorescent molecule, while DPPP=O is fluorescent. Plasma levels of hydroperoxides of phosphatidylcholine, phosphatidylethanolamine, triglycerol and cholesteryl esters have been determined by high-performance liquid chromatography (HPLC) post-column detection systems by using DPPP [12–14]. The present study was undertaken to find out if DPPP could be used to measure lipid peroxidation within cell membranes of polymorphonuclear leukocytes (PMNs) by spectrofluorophotometer and fluorescent microscopy.



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2. Materials and methods

2.1. Reagents

Dioleoyl phosphatidylcholine (DOPC), *tert*-butyl hydroperoxide (*t*BuOOH) and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma (St. Louis, MO, USA). H_2O_2 was purchased from Wako (Osaka, Japan). DPPP was obtained from Dojindo (Kumamoto, Japan). Methyl linoleate hydroperoxide (MeLOOH) was purified by HPLC from methyl linoleate obtained from Sigma and quantified using its reaction with triphenylphosphine as reported previously [15,16].

2.2. Oxidation procedures in cell-free system

Unilamellar vesicles were prepared by injecting ethanol solution (500 μl) which contained 20 mM DOPC and 1 mM DPPP into 10 ml phosphate-buffered saline (PBS, 10 mM, pH 7.4) at 37°C under stirring. The solution was further diluted with PBS and the final concentrations of DOPC and DPPP were adjusted to 20 μM and 1 μM, respectively.

The reactivity of H_2O_2, MeLOOH, and *t*BuOOH with DPPP was assayed at 37°C in chloroform/methanol (1/1) solution and in liposomal suspensions containing 10 μM peroxide and 1 μM DPPP. Oxida-

*Corresponding author. Fax: (81)-3-5452 5201.
E-mail: nonoriko@oxygen.rcast.u-tokyo.ac.jp

Abbreviations: *t*BuOOH, *tert*-butyl hydroperoxide; CCD, charge-coupled device; DCFH-DA, dichlorodihydrofluorescein diacetate; DHR, dihydrorhodamine; DMSO, dimethyl sulfoxide; DOPC, dioleoyl phosphatidylcholine; DPPP, diphenyl-1-pyrenylphosphine; DPPP=O, diphenyl-1-pyrenylphosphine oxide; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; MeLOOH, methyl linoleate hydroperoxide; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear leukocyte

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tion of DPPP was monitored every 30 s by following fluorescence for 5 min using a Perkin Elmer LB50B spectrofluorophotometer equipped with a thermostatically controlled cuvette. Wavelengths of excitation and emission were set at 351 nm (slit width 5.0 nm) and 380 nm (slit width 5.0 nm), respectively. Solution and liposomal suspension of DPPP without hydroperoxide were used as the negative controls.

2.3. Preparation of mouse PMNs

Mouse PMNs were harvested from female ICR mice (Charles River, Japan) weighing 23–28 g by lavage with PBS 5 h after i.p. injection of 2 ml of 4% thioglycollate (Difco Laboratories, Detroit, MI, USA). Cells were washed three times with PBS and adjusted at a concentration of 1×10^7 cells/ml. DPPP was dissolved in dimethylsulfoxide (DMSO) at a concentration of 5 mM. The cell suspension was incubated with 50 μM DPPP for 10 min at 37°C. Then the cells were washed twice with Hanks' balanced salt solution (HBSS) supplemented with Ca^{2+} and Mg^{2+}.

In some experiments, cells were labeled with DPPP=O instead of DPPP.

2.4. Oxidation procedures of PMNs

Various concentrations of H_2O_2 and MeLOOH were added to DPPP-containing PMNs (5×10^5 cells/ml). After incubating for 60 min at 37°C, the fluorescent intensity of the cell suspension was measured by spectrofluorophotometer under the same condition for homogeneous solution and liposomal suspension.

The oxidation in PMNs stimulated by PMA was also examined. DPPP-containing PMNs (5×10^5 cells/ml) were stimulated with 100 nM PMA and incubated at 37°C for 4 h.

2.5. Image analysis

Microscopic analysis was performed using an Axiovert fluorescence microscope (Zeiss, Oberkochen, Germany) equipped with a 100-W mercury arc lamp, connected to a black and white intensified charge-coupled device (CCD) camera. Each fluorescence image was obtained through a 40× objective lens (Olympus 1.3 NA, oil immersion). Excitation light was filtered by a 351-nm band-pass filter (16 nm band width) and the fluorescence of DPPP=O was obtained using a 365-nm dichroic mirror and 380-nm emission filter (20 nm band width). The output of the CCD camera was accumulated for 1 s.

For the image analysis, PMNs (1×10^6 cells/ml) suspended in HBSS containing 1% fetal bovine serum (FBS) were allowed to adhere in a 35-mm glass base dish (Iwaki, Japan). DPPP was dissolved in DMSO at a concentration of 5 mM and added to cells at a final concentration of 5 μM. Cells with 5 μM DPPP were incubated for 15 min and washed with HBSS containing 1% FBS to remove DPPP which was not incorporated in cells. The sample was placed on the microscope and then 5 μM MeLOOH was added as a methanol solution. The fluorescence of DPPP=O was monitored for 30 min in the same field.

3. Results

3.1. Reaction of DPPP with hydroperoxides in organic solution and liposomal membranes

DPPP is known to react with hydroperoxides to give fluorescent DPPP=O [11]. First, the reactivities of DPPP with hydroperoxides in homogeneous solution were examined. As shown in Fig. 1A, when 10 μM hydroperoxides were reacted with 1 μM DPPP in chloroform/methanol (1/1) at 37°C, the fluorescent intensity measured spectrofluorophotometrically increased linearly for 5 min. DPPP=O was formed at 3.8-, 3.3-, and 1.2-fold compared with the control without peroxides when reacted with 10 μM MeLOOH, H_2O_2 and *t*BuOOH, respectively. MeLOOH and H_2O_2 showed high reactivity toward DPPP compared with *t*BuOOH.

Liposomal membranes are often used as a model of biological membranes. DPPP incorporated into DOPC liposomal membranes was then reacted with peroxides and the increase of DPPP=O was fluorophotometrically followed (Fig. 1B). The peroxide was added as a methanol solution. When MeLOOH was added to liposomal suspensions, the formation of DPPP=O reached a plateau in 1 min and the fluorescent level was 22-fold higher than the control. H_2O_2 and *t*BuOOH caused only slight increases of DPPP=O.

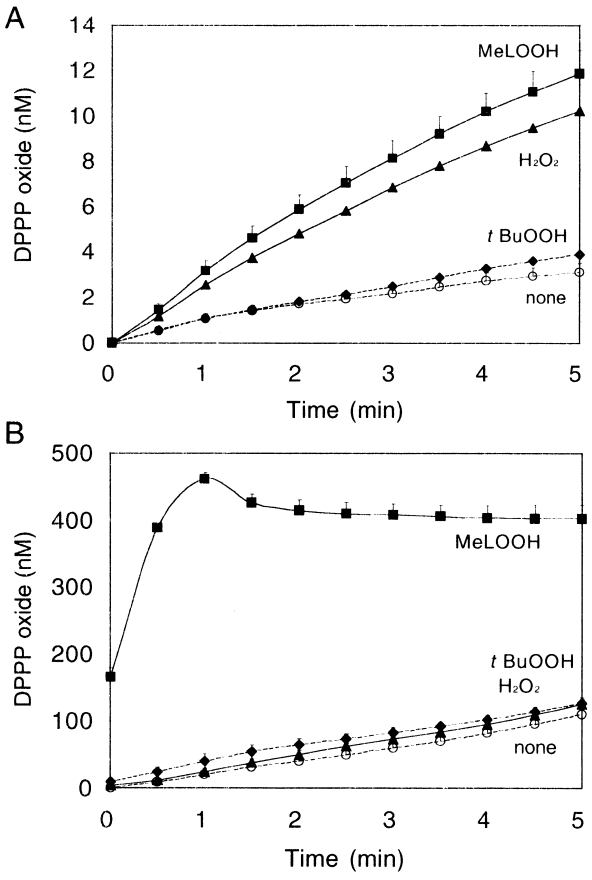

Fig. 1. DPPP oxidation by hydroperoxides in (A) chloroform/methanol (1/1) and (B) DOPC liposomal membranes. The formation of DPPP=O in the absence (none) or presence of hydroperoxide such as MeLOOH, H_2O_2, or *t*BuOOH was followed spectrofluorophotometrically. All experiments were performed at 37°C in air and the initial concentrations of DPPP and hydroperoxide were 1 μM and 10 μM, respectively. Values are expressed as mean ± S.D. ($n=3$).

DPPP was oxidized spontaneously but slowly both in homogeneous solutions and in liposomal suspensions.

3.2. Formation of DPPP=O in PMNs

The incorporation and stability of DPPP=O in PMNs were first studied. DPPP=O was incorporated into cells and then extracted with chloroform/methanol (2/1). The amount of DPPP=O incorporated in cells was fluorophotometrically quantified as about 0.35 nmol/10^6 cells using a standard curve in chloroform solution (data not shown). And when DPPP=O-containing PMNs were incubated at 37°C, the fluorescence of DPPP=O was stable in PMN for at least 4 h (data not shown).

DPPP was incorporated into PMN similarly and reacted with either H_2O_2 or MeLOOH. DPPP was oxidized by MeLOOH to give DPPP=O much more efficiently than by H_2O_2 as observed in liposomal membrane (Fig. 2). When PMNs (5×10^5 cells/ml) containing DPPP were stimulated with PMA, DPPP=O was formed and increased with time (Fig.

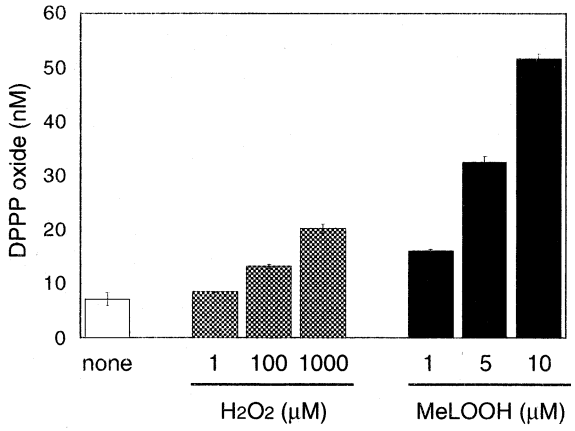

Fig. 2. Oxidation of DPPP in PMNs by H_2O_2 and MeLOOH. Mouse PMNs (1×10^7 cells/ml) were incubated with 50 μM DPPP for 10 min at 37°C and then washed and adjusted to 5×10^5 cells/ml. The indicated concentrations of H_2O_2 and MeLOOH were added and incubated at 37°C for 60 min. The amount of DPPP=O formed was measured from the increase in fluorescent intensity of the cell suspension and is expressed as mean ± S.D. ($n=3$).

3). The formation of DPPP=O was minimal without stimulation with PMA.

Microscopic observation of DPPP=O was also investigated. The increasing fluorescence was visualized microscopically at 0 and 30 min after addition of 5 μM MeLOOH (Fig. 4A,B). Little change in fluorescence was observed in 30 min without adding hydroperoxide (Fig. 4C). Staining of cells labeled with DPPP=O was clearly observed, while nuclei were not stained (Fig. 4B, inset). Unlabeled cells showed no fluorescence (data not shown).

4. Discussion

It has been reported that DPPP reacts with hydroperoxides stoichiometrically to give DPPP=O and that, due to its high fluorescence intensity, it can be applied for quantitative analysis of lipid peroxidation [11]. In fact, DPPP has been used for the measurement of the extent of oxidation in solution and in low-density lipoprotein particles [16]. In the present study,

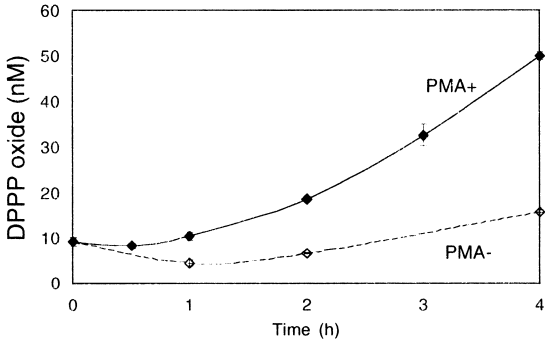

Fig. 3. Formation of lipid hydroperoxides in PMA-stimulated PMNs. Mouse PMNs (1×10^7 cells/ml) were incubated with 50 μM DPPP for 10 min at 37°C and then washed and adjusted to 5×10^5 cells/ml. PMNs were stimulated with 100 nM PMA and incubated at 37°C for 4 h. The amount of DPPP=O formed was calculated from the increase in fluorescent intensity of the cell suspension and is expressed as mean ± S.D. ($n=3$).

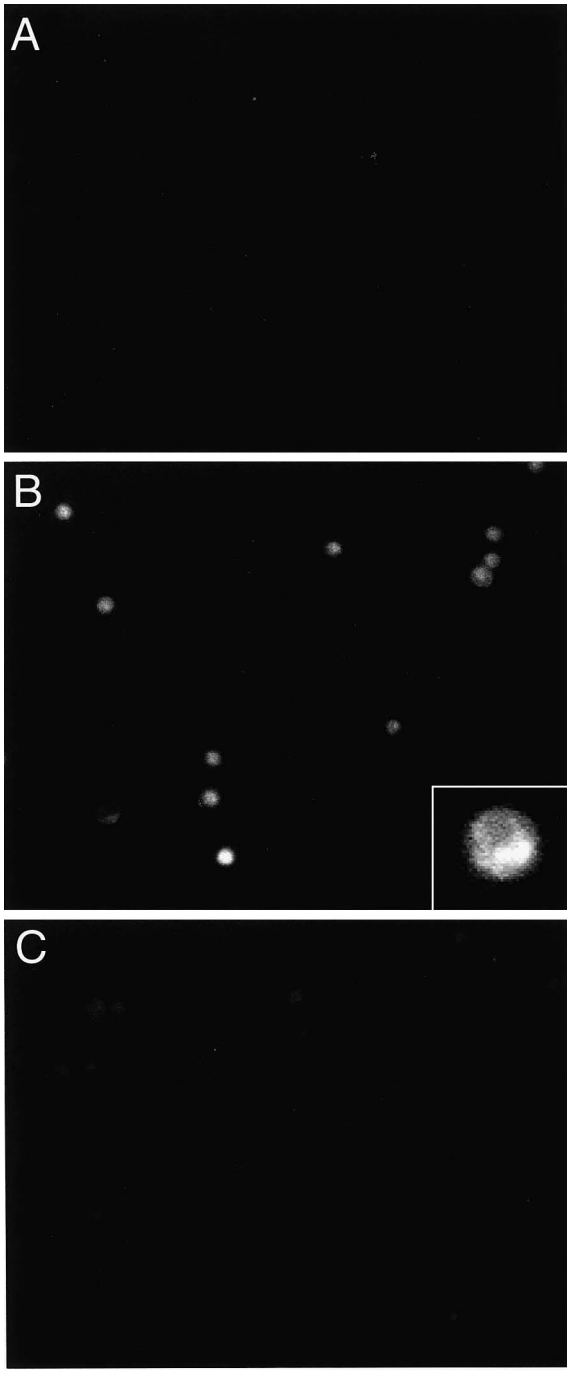

Fig. 4. Visualization of lipid peroxidation in PMNs. PMNs (1×10^6 cells/ml) were stained with 5 μM DPPP for 15 min and then washed with HBSS containing 1% FBS. Increasing fluorescence after addition of 5 μM MeLOOH (A: 0 min and B: 30 min) and without H_2O_2 (C: 30 min). Higher magnification (inset, B).

DPPP was applied to cultured cells for the first time and it is shown to be a useful probe for monitoring lipid peroxidation in the membranes.

To show that DPPP oxidation reflects the lipid peroxidation within cell membranes, we compared the reactivities of three hydroperoxides, with different polarities and solubilities, toward DPPP in both homogeneous solution and liposomal suspension. In homogeneous solution, H_2O_2 and MeLOOH almost equally oxidized DPPP although *t*BuOOH only

slightly oxidized it (Fig. 1A). However, when DPPP was reacted with hydroperoxides in DOPC liposomal membranes, MeLOOH showed much higher reactivity than both H_2O_2 and *t*BuOOH (Fig. 1B). The reactivities of hydroperoxides toward DPPP in homogeneous solution have been reported to depend on their steric hindrance effect around the hydroperoxyl group [9]. In liposomal suspensions, DPPP is supposed to localize within lipid membranes because of its high lipophilicity and reacts preferentially with lipophilic hydroperoxides such as MeLOOH.

When DPPP was added to mouse PMNs as a DMSO solution, DPPP diffused into cells and was retained in the cell membranes. Similar to the liposomal system, DPPP in cells showed remarkably high reactivity toward MeLOOH compared with H_2O_2 (Fig. 2). The low reactivity of H_2O_2 with DPPP in the membranes may be ascribed to a low local concentration of H_2O_2, although it readily crosses membranes.

We also showed that lipid peroxidation proceeded in PMNs upon stimulation with PMA (Fig. 3). PMNs are known to produce free radicals such as superoxide, hydroxyl radical, and hypochlorous acid upon stimulation [17]. The previous study using DCFH-DA showed that the oxidation proceeded in cytosol in PMA-activated PMNs [18].

The present study shows that this novel method with DPPP has several advantages. As stated above, DPPP reacts with peroxides stoichiometrically, that is, in mol-mol ratio, by a straightforward mechanism to give DPPP=O, which shows that this is suitable for quantitative measurement of lipid hydroperoxides. This is an advantage compared with the chemiluminescence method which, although sensitive, is not always applicable to quantitative analysis [19]. Secondly, DPPP is highly lipophilic and reacts with lipid hydroperoxides selectively and does not react with aqueous peroxides. This is in contrast to well-known fluorescent probes such as DCFH and DHR 123. It implies that only the lipid peroxidation taking place in the membranes can be selectively measured with DPPP. Another advantage is its high sensitivity which has been discussed previously [11–14]. In fact, it is possible to measure as low as 10^{-10} M DPPP=O with HPLC fluorescence, suggesting that 1 fmol peroxides could be quantified (unpublished data). The disadvantage of this method may be that the fluorescence emission wavelength of DPPP=O (380 nm) is near the ultraviolet region. Moreover, we examined the application of DPPP for microscopic observation. It was revealed that the use of DPPP enables the continuous observation of oxidation in cells and tissues in a non-destructive manner.

In conclusion, it was shown in the present study that DPPP is quite a useful fluorescent probe which can be applied for monitoring lipid peroxidation taking place within cell membranes.

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