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Biochimica et Biophysica Acta 1421 (1999) 131–139

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Detection of superoxide anion radical in phospholipid liposomal membrane by fluorescence quenching method using 1,3-diphenylisobenzofuran

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Received 12 May 1999; accepted 2 July 1999

Abstract

Utilization of a fluorescence dye, 1,3-diphenylisobenzofuran (DPBF) as a detector of superoxide anion radical ($O_2^{\bullet -}$) was examined. The fluorescence intensity of DPBF incorporated in phospholipid liposomes consisting of phosphatidylcholine (PC) and phosphatidylserine (PS) is effectively quenched by incubation with xanthine/xanthine oxidase system. On the other hand, xanthine or xanthine oxidase alone did not induce quenching of the DPBF fluorescence in the liposomes. Xanthine/xanthine oxidase-induced fluorescence quenching of DPBF-labeled liposomes was almost completely protected by the addition of superoxide dismutase (SOD, 1 U/ml), but not by heat-denatured SOD (10 min boiling) at the same concentration. On the other hand, catalase (1 U/ml), and hydroxyl radical and singlet oxygen scavengers (10 mM sodium benzoate, 300 mM mannitol, 1 mM tryptophan and 1 mM sodium azide) did not protect xanthine/xanthine oxidase-induced fluorescence quenching of DPBF-labeled liposomes. The concentration dependence profiles of xanthine oxidase on the DPBF fluorescence quenching and $O_2^{\bullet -}$ generation showed that there is a good correlation between these parameters. Under the present experimental conditions, approximately 7 μM H_2O_2/30 min were produced, but the addition of H_2O_2 (1 mM) to DPBF-labeled liposomes did not quench the dye fluorescence in the liposomes. Temperature dependence profiles of the DPBF fluorescence quenching induced by xanthine/xanthine oxidase treatment and the excimer fluorescence formation of pyrene molecules embedded in the liposomal membrane suggested that the quenching efficiency of the DPBF fluorescence is largely dependent on their lipid dynamics. Based on these results, we proposed the possibility that DPBF fluorescence quenching method is able to be used as a simple method for detecting $O_2^{\bullet -}$ inside the membrane lipid layer and that DPBF fluorescence quenching by $O_2^{\bullet -}$ is controlled by the physical state of membrane lipids.

Keywords: Superoxide anion radical; 1,3-Diphenylisobenzofuran; Fluorescence quenching method; Phospholipid liposome

1. Introduction

It is now generally accepted [1–5] that oxidative stress induced by generation of reactive oxygen species (ROS), such as superoxide anion radical ($O_2^{\bullet -}$), H_2O_2, hydroxyl radical and singlet oxygen, is closely related to the onset of a number of diseases including

Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; SOD, superoxide dismutase; DPBF, 1,3-diphenylisobenzofuran; *p*-NDA, *p*-nitrosodimethylaniline; BHA, 3(2)-*tert*-butyl-4-hydroxyanisole; ROS, reactive oxygen species; $O_2^{\bullet -}$, superoxide anion; NO, nitric oxide; $ONOO^-$, peroxynitrite

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0005-2736/99/$ – see front matter
PII: S0005-2736(99)00119-4

inflammatory diseases, ischemic-reperfusion injury, atherosclerosis, cancer and aging.

$O_2^{\bullet-}$ is widely generated as a reduced intermediate of molecular oxygen in a variety of biological systems by either auto-oxidation processes or by enzymes involved in aerobic metabolism, and its production is increased under conditions of oxygen stress and related phenomena [6,7]. Although $O_2^{\bullet-}$ itself is much less reactive, this radical easily turns to hydroxyl radical which is the most potent radical, through the Fenton reaction in the presence of transition metal ions such as Fe^{2+} [8]. Koch et al. [9] have suggested that the limiting step of hydroxyl radical production is $O_2^{\bullet-}$ consumption in the system. In fact, Biaglow et al. [10] have recently reported that metal ion-catalyzed production of the hydroxyl radical is dependent on the $O_2^{\bullet-}$ concentration. Furthermore, recently, increasing attention has been also focused on the interplay of nitric oxide (NO) and $O_2^{\bullet-}$. NO reacts with $O_2^{\bullet-}$ at a diffusion-controlled process to produce peroxynitrite ($ONOO^-$) and hydroxyl radical [11–13]. The endogenous production of $ONOO^-$ has been also implicated in the pathogenesis of atherosclerosis [14] and neurodegenerative diseases [15]. These findings suggest that diffusion of $O_2^{\bullet-}$ towards the production sites of hydroxyl radical and/or $ONOO^-$ in cellular membranes may be an important factor in determining the development of cellular damage relating to ROS production.

Up to this time, cytochrome *c* reduction [16,17] and nitroblue tetrazolium (NBT) reduction [18–20] methods have been widely employed for detection of $O_2^{\bullet-}$ in in vitro systems. However, recently, Gomes et al. [20] have reported that the NBT reduction method is not adequate to determination of $O_2^{\bullet-}$ concentration in phospholipid liposomes consisting of egg phosphatidylcholine, because NBT decomposes to give a complex product mixture.

Therefore, finding a sensitive and specific probe to detect ROS including $O_2^{\bullet-}$ is important for analysis of mechanisms of the onset of ROS-mediated cellular injury. The objectives of the present study are to evaluate the ability of a fluorescence dye DPBF as a probe for detecting $O_2^{\bullet-}$ and to ascertain the validity of DPBF fluorescence quenching method for the analysis of $O_2^{\bullet-}$ permeation in phospholipid liposomal membranes. This communication suggests that DPBF is a good candidate for an $O_2^{\bullet-}$ detecting probe and that the DPBF fluorescence quenching method can be used as a simple and useful method in analysis of $O_2^{\bullet-}$ permeation in the lipid layer in phospholipid membranes.

2. Materials and methods

2.1. Materials

Phosphatidylcholine (egg yolk, PC), phosphatidylserine (PS), xanthine oxidase (buttermilk, 0.82 U/mg protein), superoxide dismutase (bovine erythrocytes, 5800 U/mg protein), horseradish peroxidase (type I, 120 purpurogallin U/mg solid), catalase (bovine serum, 2900 U/mg protein), nitroblue tetrazolium (NBT), 1,3-diphenylisobenzofuran (DPBF) and pyrene were purchased from Sigma (St. Louis, MO). Xanthine monosodium salt and 3(2)-*tert*-butyl-4-hydroxyanisole (BHA) were obtained from Wako Pure Chemical Co. (Osaka, Japan). Other chemicals used in the present study were of the purest grade. Stock solutions (1 mM) of DPBF and pyrene were made by dissolving in ethanol, and stored at 4°C in the dark until use. The molecular structure of DPBF is presented in Fig. 1.

2.2. Preparation of phospholipid liposomes

Chloroform solutions of PC (10 mg/ml) and PS (1 mg/ml) were mixed, and then evaporated to dryness with a stream of nitrogen gas. The residual solvent was completely removed under vacuum, and appropriate amounts of 10 mM Tris–HCl buffer (pH 7.4) were added, then the mixture was sonicated with an Ultra Disrupter UR-200P (Tomy Seiko Co., Tokyo, Japan) until the dispersion became clear. This clear solution was then centrifuged at $25\,000 \times g$ for 20 min, and the supernatant was used in the present study.

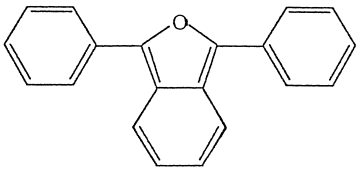


Fig. 1. The chemical structure of DPBF.

2.3. Fluorescence measurements

Fluorescence measurements were performed at 37°C using a Hitachi 850 spectrofluorimeter equipped with a rhodamine B quantum counter, unless otherwise specified. Pyrene-labeled liposomes were prepared by incubating PC/PS liposomes (1 mg/ml) with 5 μM pyrene in 30 mM Tris–HCl buffer (pH 7.4) for 10 min at 37°C, and then left to stand for 24 h at 4°C. The final concentration of ethanol in the reaction mixture was 0.3%. The monomer (I_M) and excimer (I_E) fluorescence intensities of pyrene-labeled liposomes were measured at 392 and 470 nm, respectively, using the excitation wavelength at 340 nm, and the efficiency of excimer fluorescence formation was expressed as the value of I_E/I_M.

DPBF fluorescence was measured as follows. PC/PS liposomes (0.2 mg/ml) were preincubated for 5 min at 37°C with 8.3 μM DPBF in a reaction mixture containing 30 mM Tris–HCl buffer (pH 7.4), 2 mM xanthine monosodium salt and 0.2 mM BHA in the dark, in the absence and presence of antioxidants or radical scavengers. After transferring the reaction mixture (3 ml) to a fluorescence measurement cuvette, 10 mU/ml xanthine oxidase was added, rapidly mixed, then the DPBF fluorescence was monitored (excitation 410 nm, emission 455 nm) [21]. The extent of DPBF fluorescence quenching expressed as the difference (ΔFI) between the fluorescence intensities at time 0 (before addition of xanthine oxidase) and at time t (t = minutes after start of incubation with the enzyme). In determination of the pseudo-first-order rate constant (k') of xanthine/xanthine oxidase-induced quenching of the DPBF fluorescence, the fluorescence intensity of DPBF-labeled liposomes was measured within 3 min after the addition of xanthine oxidase to the reaction mixture, because the fluorescence change of the labeled liposomes linearly proceeded until 5 min after the addition of the enzyme. The fluorescence intensity of DPBF in ethanol was proportional to the DPBF concentration up to 16 μM (data not shown).

2.4. Determination of the amount of DPBF

The incorporated amounts of the fluorescence dye into the liposomes were determined by a filtration method as follows. The reaction mixture was filtered through a DISMIC-25 syringe filter (0.2 μm pore size; Toyo Roshi, Tokyo, Japan) and then the absorbance at 410 nm of the original reaction mixture and the filtrate were measured [22]. Under the present experimental conditions, 25–30% (2.1–2.5 μM) of the dye employed were incorporated into the liposomes.

2.5. Measurements of reactive oxygen species

$O_2^{\bullet-}$ was generated by a xanthine/xanthine oxidase system and measured by the NBT reduction method as follows. PC/PS liposomes (0.2 mg/ml) were preincubated for 2 min at 37°C with 2 mM xanthine monosodium salt and 1 mM NBT in 30 mM Tris–HCl buffer (pH 7.4). The reaction was started by the addition of xanthine oxidase (10 mU/ml), and monitored spectrophotometrically at 560 nm ($\Delta\varepsilon = 16\,500$ $M^{-1}\,cm^{-1}$ [19]) during 30 min unless otherwise specified. In kinetic analysis of $O_2^{\bullet-}$ production, the substrate concentration was varied to 100 μM and the absorbance at 560 nm was recorded until 3 min after of the addition of the enzyme, because the absorbance change proceeded linearly up to 5 min. The total volume of the reaction mixture was 3 ml. Hydroxyl radical and H_2O_2 production were measured by the *p*-nitrosodimethylaniline (*p*-NDA) bleaching method [23] and the 4-aminoantipyrine/peroxidase method [24], respectively. In the former, PC/PS liposomes (0.2 mg/ml) was preincubated for 2 min at 37°C in a solution containing 50 mM NaCl, 2 mM xanthine monosodium salt, 0.2 mM BHA and 50 μM *p*-NDA. The reaction was started by the addition of xanthine oxidase (10 mU/ml) to the reaction mixture (3 ml), and the absorbance recorded at 440 nm. On the other hand, H_2O_2 production was measured using 2.0 mg/ml of PC/PS liposomes as a final concentration, and other experimental conditions were the same as those described in the *p*-NDA bleaching method, except for the use of 30 mM Tris–HCl buffer (pH 7.4) in place of NaCl. The total volume of the reaction mixture was 3 ml. The amount of H_2O_2 produced was calculated using the molar extinction coefficient of 6400 $M^{-1}\,cm^{-1}$ [24].

2.6. Uric acid determination

The formation of uric acid from xanthine by xan-

thine oxidase treatment was measured according to the procedure described by Fridovich [25] as follows. The reaction was started by the addition of xanthine oxidase (10 mU/ml) to the reaction mixture containing 100 μM xanthine monosodium salt and 30 mM Tris–HCl buffer (pH 7.4) at 37°C and the absorbance at 295 nm was recorded during 3 min, because the absorbance change proceeded linearly up to 3 min after the addition of the enzyme. The amount of uric acid formed was calculated using the molar extinction coefficient ($\varepsilon = 11\,000\ M^{-1}\,cm^{-1}$) at 295 nm [25].

2.7. Statistical analysis

Data are presented as mean ± S.D. for three independent measurements. To determine statistical significance between groups, the data were analyzed by an ANOVA Bonferroni's multiple *t*-test.

3. Results

3.1. Fluorescence characteristics of DPBF

As shown in Fig. 2, incubation of PC/PS liposomes with DPBF for 30 min at 37°C resulted in a marked fluorescence exhibiting two peaks at 455 and 477 nm in the emission spectrum. A similar spectrum with a high fluorescence intensity of the dye was also observed when the dye was dissolved in ethanol. On the other hand, sonication (5 min at 0°C) of DPBF-labeled liposomes caused a marked decrease of the dye fluorescence. In addition, the fluorescence intensity of the dye was not detected in the buffer solution without the liposomes.

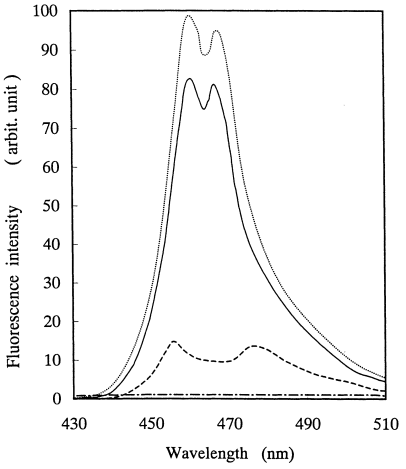


Fig. 2. Fluorescence spectra of DPBF under various conditions at 37°C in 30 mM Tris–HCl buffer (pH 7.4). The fluorescence spectra of DPBF were recorded with the excitation wavelength at 410 nm. The concentration of PC/PS liposomes was 0.2 mg/ml. Solid line, PC/PS liposomes; dashed line, sonicated PC/PS liposomes; dot-dashed line, Tris–HCl buffer (pH 7.4); dotted line, ethanol (95 v/v%).

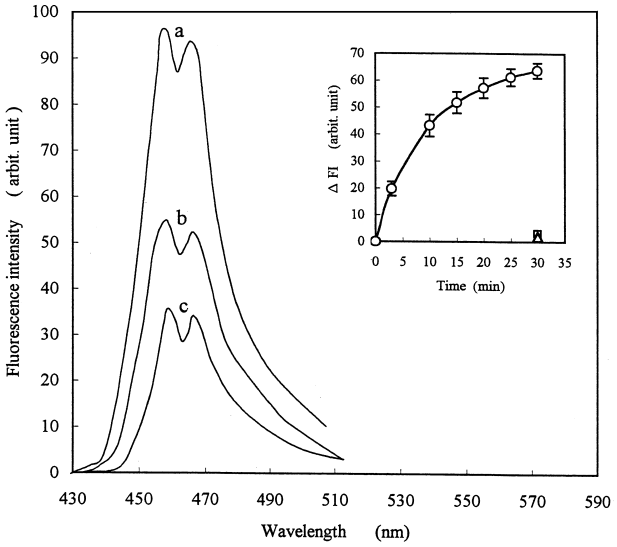
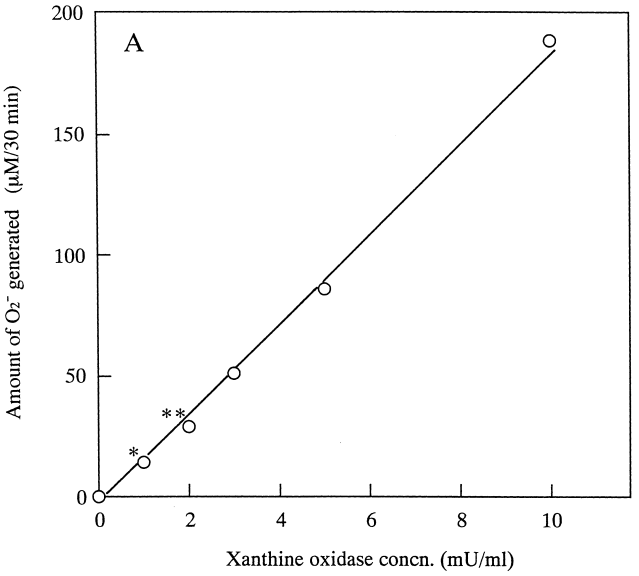


Fig. 3. Time course of xanthine/xanthine oxidase-induced fluorescence quenching of DPBF-labeled liposomes. a, time 0 (no addition of xanthine oxidase); b, 10 min after start of incubation; c, 30 min after start of incubation. The fluorescence intensity (arbit. unit) described in the inset was expressed as ΔFI (see Section 2). Other experimental conditions are the same as those described in the legend to Fig. 1. (○) Complete system (2 mM xanthine/10 mU/ml xanthine oxidase); (△) 10 mU/ml xanthine oxidase alone; (□) 2 mM xanthine alone.

3.2. Change in DPBF fluorescence by xanthine/xanthine oxidase treatment

When DPBF-labeled liposomes were incubated with xanthine oxidase in the presence of xanthine at 37°C, the dye fluorescence was quenched in a time-dependent manner (Fig. 3). On the other hand, incubation of the labeled liposomes with either xanthine or xanthine oxidase alone did not cause any change in the DPBF fluorescence, indicating that the fluorescence change in DPBF-labeled liposomes induced by xanthine/xanthine oxidase treatment is



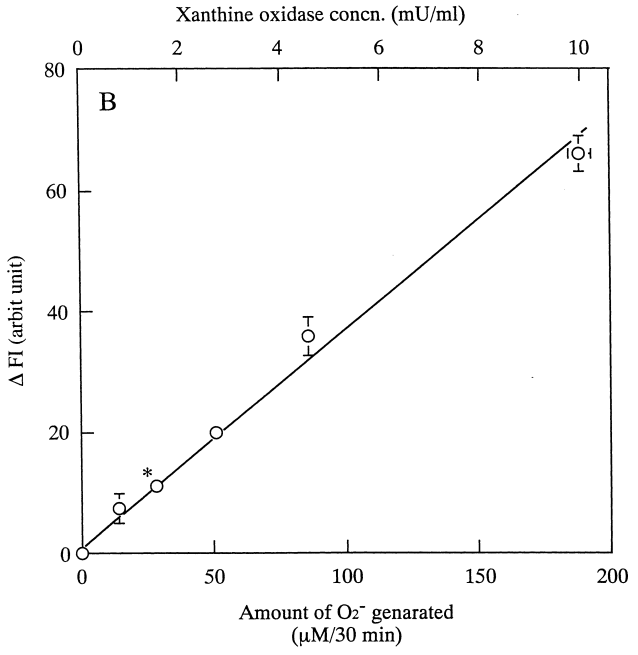


Fig. 4. Concentration dependence of xanthine oxidase on $O_2^{\bullet-}$ generation and DPBF fluorescence quenching. The concentration of xanthine oxidase was varied from 1 to 10 mU/ml. The amount of $O_2^{\bullet-}$ (A) and the DPBF fluorescence (B) were measured after 30 min of the addition of xanthine oxidase in the presence of xanthine (2 mM), and the extent of DPBF fluorescence quenching was expressed as ΔFI as described in the legend to Fig. 3. The data of $O_2^{\bullet-}$ generation described in B were obtained from A. Other experimental conditions are the same as those described in the legend to Fig. 2. (A) $*P < 0.05$ vs. the system without xanthine oxidase, $**P < 0.05$ vs. the system with xanthine oxidase (1 mU/ml). (B) $*P < 0.05$ vs. the system with xanthine oxidase (1 mU/ml).
←

not due to interaction of the liposomes with xanthine or xanthine oxidase itself. In addition, under the present experimental conditions, the presence of 0.2 mM BHA in the reaction mixture showed no effect on the fluorescence response against xanthine/xanthine oxidase treatment (data not shown).

As can be seen in Fig. 4A and B, the concentration dependence of xanthine oxidase on $O_2^{\bullet-}$ generation showed that $O_2^{\bullet-}$ was almost linearly produced in the concentration range of the enzyme tested, and that there is a good correlation between the extent of DPBF fluorescence quenching and the amount of $O_2^{\bullet-}$ generated.

3.3. Effects of SOD and other radical scavengers

The effects of increasing concentrations of SOD on xanthine/xanthine oxidase-induced DPBF fluorescence quenching were examined.

As shown in Fig. 5, the fluorescence quenching of DPBF-labeled liposomes induced by xanthine/xanthine oxidase treatment effectively protected by the addition of SOD in a concentration-dependent manner, and the protective effect of SOD was almost saturated above 0.7 U/ml of the enzyme. On the other hand, heat-denatured SOD (boiled for 10 min) with a consequent loss of 92% of its activity did almost not protect the DPBF fluorescence quenching induced by xanthine/xanthine oxidase treatment, and the ΔFI values of DPBF-labeled liposomes without and with the denatured SOD (1 U/ml) after incubation with xanthine oxidase for 30 min at 37°C were 63.8 ± 2.8 and 63.3 ± 4.8 ($n = 3$), respectively. The addition of SOD (1 U/ml) to DPBF-labeled liposomes in the absence of xanthine oxidase did also not affect on the fluorescence intensity of the dye (data not shown).

On the other hand, scavengers of hydroxyl radical and singlet oxygen (sodium benzoate, mannitol, tryptophan and sodium azide) and catalase showed no protective effect on xanthine/xanthine oxidase-induced DPBF fluorescence quenching (Table 1). In the absence of xanthine/xanthine oxidase system, these compounds and the enzyme did not induce any change in the DPBF fluorescence in the lipo-

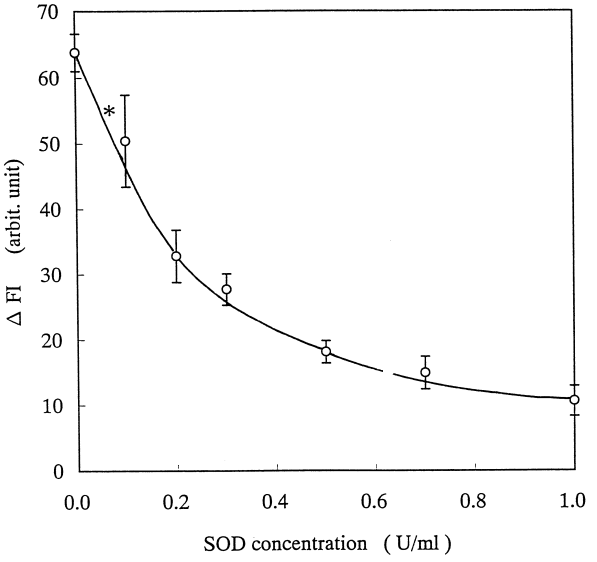


Fig. 5. Effects of increasing concentrations of SOD on DPBF fluorescence. The concentration of SOD was varied from 0.1 to 1 U/ml. The DPBF fluorescence was measured after 30 min of the addition of xanthine oxidase and expressed as ΔFI. Other experimental conditions were the same as those described in the legend to Fig. 2. *$P < 0.05$ vs. the system without SOD.

somes (data not shown). Furthermore, the addition of H_2O_2 to DPBF-labeled liposomes in the absence of xanthine/xanthine oxidase system did also not induce quenching of the dye fluorescence ($\Delta FI = 63.8 \pm 2.8$ and 63.5 ± 3.2, for the systems without and with 1 mM H_2O_2, respectively: $n = 3$), indicating that H_2O_2 is not involved in xanthine/xanthine oxidase-induced quenching of the DPBF fluorescence in the liposomes observed in the present study.

Table 1
Effects of several radical scavengers or antioxidant on DPBF fluorescence quenching in PC/PS liposomes

Scavengers	Concentration	ΔFI (arbitrary unit)
No addition	–	63.8 ± 2.8
Mannitol	300 mM	58.0 ± 6.9
Sodium benzoate	10 mM	57.5 ± 4.1
Tryptophan	1 mM	61.5 ± 2.2
Sodium azide	1 mM	62.2 ± 4.5
Catalase	1 U/ml	63.0 ± 5.1

The liposomes were incubated with xanthine/xanthine oxidase for 30 min at 37°C in the presence and absence of radical scavengers or antioxidant. Other experimental conditions are the same as those described in the legend to Fig. 2.

3.4. Effects of temperature variation

To investigate the contribution of the lipid dynamics on DPBF fluorescence quenching in PC/PS liposomes induced by xanthine/xanthine oxidase treatment, the effects of temperature variation on the efficiency of the excimer fluorescence formation of pyrene-labeled PC/PS liposomes and the rate of the

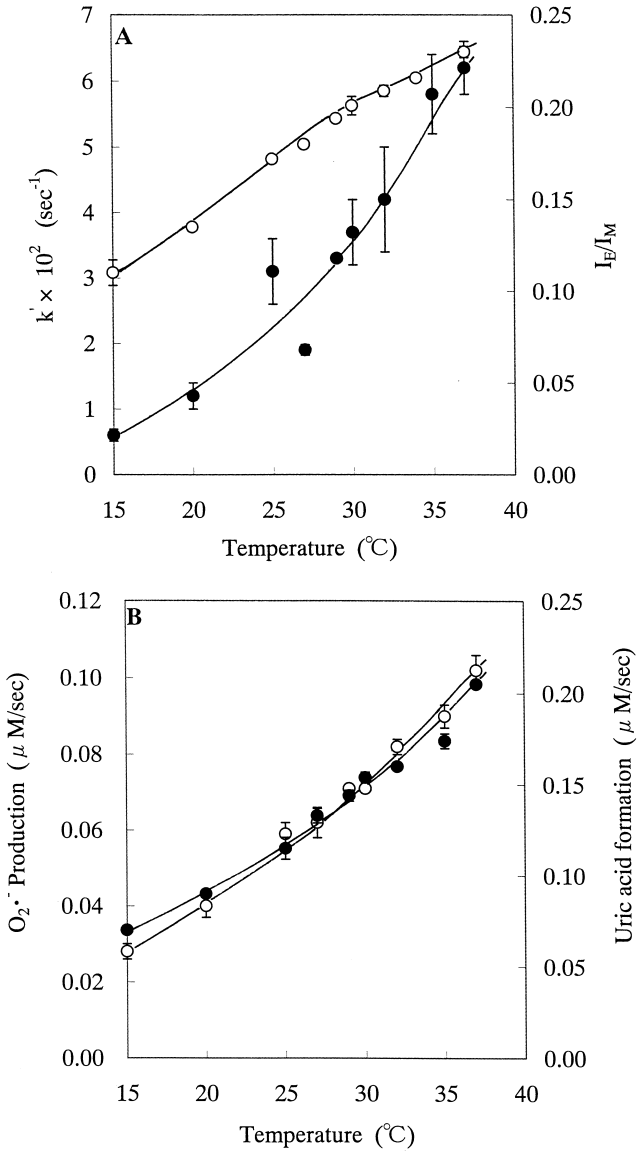


Fig. 6. Effects of temperature variation on pyrene excimer fluorescence, DPBF fluorescence and xanthine oxidase activity. Temperature was varied from 15°C to 37°C. (A) Pyrene excimer formation (○) and DPBF fluorescence quenching (●). (B) Uric acid formation (○) and $O_2^{\cdot-}$ production (●).

fluorescence quenching of DPBF-labeled liposomes were examined.

When the temperature was increased, the efficiency of excimer fluorescence formation of pyrene molecules embedded in the liposomal membranes and the pseudo-first-order rate constant (k') of xanthine/xanthine oxidase-induced quenching of the DPBF fluorescence in the liposomes were increased depending on the temperature with the similar bending points (27–30°C, Fig. 6A). In addition, the temperature dependence profiles of the enzyme activities showed that uric acid formation and $O_2^{\bullet-}$ generation from xanthine by xanthine oxidase treatment in the absence of the liposomes were also transitionally enhanced around 27–30°C (Fig. 6B), indicating that the enzyme is not denatured in the temperature range tested.

4. Discussion

DPBF has been widely employed as a singlet oxygen trap in organic solutions [22] and in phospholipid liposomes [21,26]. In this paper, we proposed the possibility that the DPBF quenching method is able to be used as a simple method to detect $O_2^{\bullet-}$ in model phospholipid membranes. This method is based on the fluorescence quenching of the dye incorporated in the liposomal membranes as a consequence of reaction of DPBF with $O_2^{\bullet-}$.

Incubation of PC/PS liposomes with DPBF resulted in a marked fluorescence development of the dye. However, the DPBF fluorescence was not detected when the dye was dispersed in Tris–HCl buffer (pH 7.4), and sonication of the labeled liposomes also caused a marked decrease in the dye fluorescence (Fig. 2). In addition, it was found that 80–83% of the incorporated DPBF were excluded in the medium by sonication of the labeled liposomes. These results suggest that the dye molecules come out from the membrane lipids to aqueous phase by destruction of the membrane structure by sonication, because DPBF easily forms a non-fluorescent dimer in water [22]. From these findings, it seems that DPBF molecules are located in the hydrocarbon core in the liposomal membrane lipids and that the complete membrane structure is necessary for development of the dye fluorescence.

We found that the DPBF fluorescence in the liposomes was sensitively quenched in a time-dependent manner in the presence of both xanthine and xanthine oxidase (Fig. 3). This fluorescence quenching was markedly suppressed if SOD was added to the reaction mixture, depending on the concentration of the enzyme (Fig. 5). On the other hand, xanthine or xanthine oxidase itself did not show any change in the DPBF fluorescence. In addition, the extent of the fluorescence quenching of DPBF incorporated in the liposomes by xanthine/xanthine oxidase treatment was dependent on the extent of $O_2^{\bullet-}$ generation (Fig. 4B) and the SOD concentration (Fig. 5). From these results, it is suggested that change in the DPBF fluorescence induced by incubation of DPBF-labeled liposomes with xanthine oxidase in the presence of xanthine is due to interaction of the dye molecules embedded in the lipid layer in the liposomal membranes with $O_2^{\bullet-}$ generated during the reaction, although the exact mechanism of the fluorescence quenching is unclear at present. This interpretation was further confirmed from the finding that heat-denatured SOD or catalase did not protect quenching of the DPBF fluorescence induced by xanthine/xanthine oxidase treatment.

It is well known [25] that the reaction of xanthine and xanthine oxidase under aerobic conditions results in the production of both univalent and divalent reductions of oxygen, $O_2^{\bullet-}$ and H_2O_2. In fact, under the present experimental conditions, 6.9 ± 0.1 μM H_2O_2 ($n = 3$) was produced by incubation of PC/PS liposomes with xanthine/xanthine oxidase for 30 min at 37°C. However, xanthine/xanthine oxidase-induced quenching of the DPBF fluorescence in the liposomes was not protected by the addition of catalase to the reaction mixture (Table 1). In addition, treatment of DPBF-labeled liposomes with H_2O_2 (1 mM) did not cause the fluorescence change. From these results, it is suggested that H_2O_2 is not involved in the fluorescence quenching of DPBF-labeled liposomes induced by xanthine/xanthine oxidase treatment.

The extent of *p*-NDA bleaching did not change through incubation of PC/PS liposomes with xanthine/xanthine oxidase ($A_{440} = 1.38 \pm 0.02$ and 1.29 ± 0.01, $n = 3$, for the systems before and 30 min after the start of treatment, respectively), indicating that hydroxyl radical is not generated under the

present experimental conditions. Britigan et al. [27] have also reported that hydroxyl radical does not appear to be a product of the oxidation of xanthine by xanthine oxidase. Therefore, the contribution of hydroxyl radical against xanthine/xanthine oxidase-induced quenching of the DPBF fluorescence observed in the present study was ruled out. In fact, several hydroxyl radical scavengers (sodium benzoate, mannitol and tryptophan) did not protect xanthine/xanthine oxidase-induced quenching of the DPBF fluorescence (Table 1). In addition, the possibility that singlet oxygen is produced from the reaction between $O_2^{\bullet-}$ and hydroperoxyl radical ($HO_2^{\bullet}$) under the present experimental conditions was also ruled out, because xanthine/xanthine oxidase-induced DPBF fluorescence quenching did not protect by the presence of singlet oxygen scavengers, tryptophan and sodium azide [28–30].

The efficiency of excimer fluorescence formation of pyrene-labeled liposomes increased depending on the temperature (Fig. 6A). Since pyrene is predominantly located in the central methylene region of aliphatic chains of the PC bilayer [31] and the excimer formation of pyrene is dependent on the solvent viscosity and the dye concentration, i.e., diffusion-controlled process [32], an increased excimer fluorescence formation of pyrene-labeled liposomes with an increase of the temperature plausibly explains an arrangement of concentrated pyrene molecules favorable to excimer formation due to an increase of the membrane lipid fluidity. In addition, the excimer formation of the pyrene-labeled liposomes was transitionally enhanced at 29–30°C, suggesting that lipid organization in the liposomal membranes is transitionally changed around this temperature.

The kinetic studies of the DPBF fluorescence showed that the rate of xanthine/xanthine oxidase-induced fluorescence quenching of DPBF-labeled liposomes was also accelerated depending on the increase of the temperature (Fig. 6A). It is of interest that the extent of the quenching rate constant (k') of the DPBF fluorescence in the liposomes by $O_2^{\bullet-}$ also markedly increased above 27°C. One possible explanation for an abrupt increase of the k' value above 27°C is a facilitated penetration of $O_2^{\bullet-}$ into the lipid layer in the liposomal membranes due to an increased $O_2^{\bullet-}$ generation, because the temperature dependence of xanthine oxidase activity, assessed by uric acid formation and $O_2^{\bullet-}$ production, in the absence of the liposomes showed a similar curve with the transition point at 27–30°C in the temperature range tested (Fig. 6B). An increased concentration of $O_2^{\bullet-}$ around DPBF molecules located in the membrane lipids may induce an enhanced quenching of the dye fluorescence. This interpretation is further supported from the finding that the extent of DPBF fluorescence quenching proceeds linearly to the $O_2^{\bullet-}$ concentration (Fig. 4B). Furthermore, it seems that the transition temperature of $O_2^{\bullet-}$ penetration is close to that of excimer fluorescence formation of pyrene molecules embedded in the membrane lipids (27–30°C). This phenomenon suggests that the efficiency of DPBF fluorescence quenching by $O_2^{\bullet-}$ may be controlled by the lipid organization in the membrane.

Based on these results, we proposed the possibility that DPBF can be used as a fluorescence indicator to detect $O_2^{\bullet-}$ in model membranes, and suggested that the lipid dynamics is the important factor in controlling $O_2^{\bullet-}$ penetration into their lipid layer, although further detailed experiments using several biological membranes and cells are necessary.

Acknowledgements

This work was partly supported by a Grant-in-Aid (no. 10672070) from the Ministry of Education, Science and Culture of Japan, and The Specific Research Fund of Hokuriku University.

References

[1] P.A. Cerutti, Prooxidant states and tumor promotion, Science 227 (1985) 375–381.
[2] B. Hennig, C.K. Chow, Lipid peroxidation and endothelial cell injury: implications in atherosclerosis, Free Radic. Biol. Med. 4 (1988) 99–106.
[3] R.A. Floyd, Role of oxygen free radicals in carcinogenesis and brain ischemia, FASEB J. 4 (1990) 2587–2597.
[4] D.R. Crawford, J.S. Greenberger, Active oxygen transforms murine myeloid progenitor cells in vitro, Int. J. Cancer 49 (1991) 744–749.
[5] B.N. Ames, M.K. Shigenaga, J.M. Hagen, Oxidants, antioxidants, and the degenerative diseases of aging, Proc. Natl. Acad. Sci. USA 90 (1993) 7915–7922.

[6] I. Fridovich, Superoxide radical and superoxide dismutase, Acct. Chem. Res. 5 (1972) 321–326.
[7] B.M. Babior, R.S. Kipnes, J.T. Curnutte, Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent, J. Clin. Invest. 52 (1973) 741–744.
[8] S.J. Stohs, D. Bagchi, Oxidative mechanisms in the toxicity of metal ions, Free Radic. Biol. Med. 18 (1995) 321–336.
[9] C.J. Koch, R.L. Howell, J.E. Biaglow, Ascorbate anion potentiates cytotoxicity of nitroaromatic compounds under hypoxic and anoxic conditions, Br. J. Cancer 39 (1979) 321–329.
[10] J.E. Bigalow, A. Kachur, Y. Manevich, Effect of purine nucleoside phosphates on OH-radical generation by reaction of Fe^{2+} with oxygen, Free Radic. Res. 26 (1997) 399–408.
[11] J.S. Beckman, T.W. Beckman, J. Chen, P.A. Marshall, A. Freeman, Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide, Proc. Natl. Acad. Sci. USA 87 (1990) 1620–1624.
[12] J.P. Bolanos, S.J.R. Heales, J.M. Land, J.B. Clark, Effect of peroxynitrite on the mitochondrial respiratory chain: differential susceptibility of neurons and astrocytes in primary culture, J. Neurochem. 64 (1995) 1965–1972.
[13] S.S. Gross, M.S. Wolin, Nitric oxide: pathophysiological mechanisms, Annu. Rev. Physiol. 57 (1995) 739–769.
[14] J.S. Beckman, W.H. Koppenol, Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly, Am. J. Physiol. 271 (1996) C1424–C1437.
[15] J.S. Beckman, The double-edged role of nitric oxide in brain function and superoxide-mediated injury, J. Rev. Physiol. 15 (1991) 53–59.
[16] R.E. Lynch, I. Fridovich, Effects of superoxide on the erythrocyte membrane, J. Biol. Chem. 253 (1978) 1838–1845.
[17] R. Gennaro, D. Romeo, The release of superoxide anion from granulocytes: effect of inhibitors of anion permeability, Biochem. Biophys. Res. Commun. 88 (1979) 44–49.
[18] M. Nishikimi, N.A. Roa, K. Yagi, The occurrence of superoxide anion in the reaction of reduced phenazine methsulfate and molecular oxygen, Biochem. Biophys. Res. Commun. 46 (1972) 849–854.
[19] W.S. Thayer, Superoxide-dependent and superoxide-independent pathways for reduction of nitroblue tetrazolium in isolated rat cardiac myocytes, Arch. Biochem. Biophys. 276 (1990) 139–145.
[20] L.F. Gomes, I.M. Cuccovia, H. Chaimovich, D.H.G.P. Barbieri, M.J. Politi, Permeation of superoxide anion through the bilayer of vesicles of a synthetic amphiphile, Biochim. Biophys. Acta 1152 (1993) 78–82.
[21] M. Wozniak, F. Tanfani, E. Bertoli, G. Zolese, J. Antosiewicz, A new fluorescence method to detect singlet oxygen inside phospholipid model membranes, Biochim. Biophys. Acta 1082 (1991) 94–100.
[22] P.B. Merkel, D.R. Kearns, Radiationless decay of singlet molecular oxygen in solution. An experimental and theoretical study of electronic-to-vibrational energy transfer, J. Am. Chem. Soc. 94 (1972) 7244–7253.
[23] G. Minotti, S.D. Aust, The requirement for iron (III) in the initiation of lipid peroxidation by iron (III) and hydrogen peroxide, J. Biol. Chem. 262 (1987) 1098–1104.
[24] M.J. Green, H.A.O. Hill, Chemistry of dioxygen, Methods Enzymol. 105 (1984) 3–22.
[25] I. Fridovich, Quantitative aspects of the production of superoxide anion radical by milk xanthine oxidase, J. Biol. Chem. 245 (1970) 4053–4057.
[26] K. Fukuzawa, K. Matsuura, A. Tokumura, A. Suzuki, J. Terao, Kinetics and dynamics of singlet oxygen scavenging by alpha-tocopherol in phospholipid model membranes, Free Radic. Biol. Med. 22 (1997) 923–930.
[27] B.E. Britigan, S. Pou, G.M. Rosen, D.M. Lilleg, G.R. Buettner, Hydroxyl radical is not a product of the reaction of xanthine oxidase and xanthine. The confounding problem of adventitious iron bound to xanthine oxidase, J. Biol. Chem. 265 (1990) 17533–17538.
[28] J. Piatt, J. O'Brien, Singlet oxygen formation by a peroxidase, H_2O_2 and halide system, Eur. J. Biochem. 93 (1979) 323–332.
[29] J.B. Feix, B. Kalyanaraman, C.F. Chignell, R.D. Hall, Direct observation of singlet oxygen production by merocyanine 540 associated with phosphatidylcholine liposomes, J. Biol. Chem. 263 (1988) 17247–17250.
[30] M. Wozniak, F. Tanfani, E. Bertoli, G. Zolese, J. Antosiewicz, A new fluorescence method to detect singlet oxygen inside phospholipid mode membranes, Biochim. Biophys. Acta 1082 (1991) 94–100.
[31] F. Podo, J.K. Blasie, Nuclear magnetic resonance studies of lecithin bimolecular leaflets with incorporated fluorescent probes, Proc. Natl. Acad. Sci. USA 74 (1977) 1032–1036.
[32] H.J. Pownall, L.C. Smith, Viscosity of the hydrocarbon region of micelles. Measurement by excimer fluorescence, J. Am. Chem. Soc. 95 (1973) 3136–3140.