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Oxygen concentration dependence of lipid peroxidation and lipid-derived radical generation: Application of profluorescent nitroxide switch

FUMIYA MITO[1#], KANA KITAGAWA[1#], TOSHIHIDE YAMASAKI[1], CHISATO SHIRAHAMA[1], TAKETOSHI OISHI[1], YUKO ITO[1], MAYUMI YAMATO[2] & KEN-ICHI YAMADA[1]

[1]*Department of Bio-functional Science, Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582, Japan, and* [2]*Innovation Center for Medical Redox Navigation, Kyushu University, Fukuoka 812-8582, Japan*

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Abstract

Lipid-derived radicals and peroxides are involved in the pathogenesis of oxidative stress diseases and, although lipid peroxide production is a required reaction between a lipid radical and molecular oxygen, a useful lipid radical detection method has remained tentative. Also, the effect of oxygen concentration on lipid peroxide production must be considered because of the hypoxic conditions in cancer and ischemic regions. In this study, the focus was on nitroxide reactivity, which allows spin trapping with carbon-centred radicals via radical–radical reactions and fluorophore quenching through interactions with nitroxide's unpaired electron. Thus, the aim here was to demonstrate a useful detection method for lipid-derived radicals as well as to clarify the effects of oxygen concentration on lipid peroxide production using profluorescent nitroxide. This latter compound reacted with lipid-derived radicals in a manner inversely dependent on oxygen concentration, resulting in fluorescence due to alkoxyamine formation and, conversely, lipid peroxide concentrations decreased with lower oxygen in the reaction system. Furthermore, nitroxide inhibited lipid peroxide production and stopped oxygen consumption in the same solution. These results suggested that the novel application of profluorescent nitroxide could directly and sensitively detect lipid-derived radicals and that radical and peroxide production were dependent on oxygen concentration.

Keywords: *Nitroxide, lipid radical, lipid peroxide, oxygen, fluorescence.*

Abbreviations: *ESR, electron spin resonance; dansyl chloride, 5-(dimethylamino)naphthalene-1-sulphonyl chloride; dansyl-TEMPO, 4-[5-(dimethylamino)-1-naphthalenesulphonamido]-2,2,6,6-tetramethylpiperidine-1-oxyl; LC/MS, liquid chromatography/mass spectroscopy; pO_2, partial oxygen pressures; TBARS, thiobarbituric acid reactive substance; PBN, N-tert-butyl-α-phenylnitrone; PUFAs, polyunsaturated fatty acids.*

Introduction

Free radicals and reactive oxygen species contribute to the maintenance of biological homeostasis [1]. However, an excess of generated free radicals can readily attack biological molecules, such as lipids, proteins and DNA, leading to subsequent functional losses [2]. Recently, the relationship between free radical production and the pathogenesis of oxidative diseases has received attention [3,4]. Oxygen molecules also participate in radical chain reactions, producing lipid peroxides, carbonylated proteins and 8-hydroxydeoxyguanosine. In particular, free radical mediated peroxidation of the polyunsaturated fatty acid (PUFA) constituents of biomembranes has been focused upon as the cause of atherosclerosis [5]. The related chain reaction proceeds by the following scheme [6,7].

$$LH + R^{\bullet} \rightarrow L^{\bullet} + RH \quad (1)$$
$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \quad (2)$$
$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet} \quad (3)$$

#These authors contributed equally to this work.
Correspondence: Ken-ichi Yamada, PhD, Department of Bio-functional Science, Faculty of Pharmaceutical Sciences, Kyushu University, 3-1-1 Higashi-ku, Maidashi, Fukuoka 812-8582, Japan. Tel: +81-92-642-6624. Fax: +81-92-642-6626. E-mail: yamada@pch.phar.kyushu-u.ac.jp

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showing (1) hydrogen atom transfer from PUFA (LH) to a chain initiating radical ($R^{\bullet}$), (2) reaction of the lipid radical ($L^{\bullet}$) with molecular oxygen to yield a chain-carrying lipid peroxyl radical ($LOO^{\bullet}$) and (3) hydrogen atom transfer from PUFA to the lipid peroxyl radical to produce lipid peroxide (LOOH) and $L^{\bullet}$. In addition, lipid peroxides decompose into lipid, peroxyl and alkoxyl radicals [8]. Once formed, peroxyl radicals can rearrange via a cyclization reaction to form malondialdehyde and 4-hydroxynonenal, two major aldehyde products which can exert cytotoxic and genotoxic effects [9]. Lipid peroxidation products can act as signalling messengers [10] and, as peroxides are unstable, they can subsequently undergo reactions as substrates of various enzymes. Meanwhile, the oxygen partial pressure (pO_2) of a diseased site, such as in cancer and chronic inflammatory disease, is lower compared with a normal site and, thus, hypoxia adaptation is required. Oxygen tension regulates many physiologically important gene expressions, such as hypoxia-inducible factor-1 and vascular endothelial growth factor [11,12]. The present study focused on the oxygen content in reaction systems; higher oxygen favouring completion of the lipid peroxidation process and production of lipid peroxide and lower oxygen suppressing the process and generating it. According to oxygen concentration, methyl linoleate is oxidized and produces peroxide [13]. Further, elevated oxygen content during ischemia accelerated lipid peroxidation and resulted in tissue injury [14]. Thus, the amounts of lipid-derived radicals and peroxides would be modulated by a target region's oxygen concentration. Although the detection of highly reactive, lipid-derived radicals is challenging, the development of useful detection systems would be helpful in the analysis of biological free radical reactions and also to accurately understand the pathogenesis of oxidative diseases.

Nitroxides with an unpaired electron are stable free radical compounds, widely used as antioxidants [15–17] and spin probes [18–20]. Based on the reaction mechanism, the radical–radical reaction between nitroxide and a carbon-centred radical produce alkoxyamine derivatives (>N-O-R) [21,22]. This function provides the possibility of identifying protein-based, carbon-centred radicals using liquid chromatography/mass spectroscopy (LC/MS) techniques [23]. Furthermore, a sensitive and novel approach, employing covalent coupling of nitroxide with a fluorophore, leads to intramolecular quenching of fluorescence emission by electron exchange interactions [24]. If the unpaired electron of a nitroxide radical reacts with a free radical, fluorescence may recover (Figure 1A). Although these fluorophore–nitroxide coupled systems have been applied as highly sensitive optical probes for a redox environment [25], vitamin C [26] and carbon-centred radicals [27], detection of lipid-derived radical by this systems has not been developed. It was expected that, at lower oxygen tension, pro-fluorescent nitroxide would effectively compete with the oxygen molecule and sensitively detect a lipid-derived radical. The aim of the present study was to clarify the effect of oxygen concentration on lipid peroxide and lipid-derived radical generation using pro-fluorescent nitroxide switching methods and the results clearly suggested that lower oxygen decreased lipid peroxide concentrations. Also, pro-fluorescent nitroxide successfully detected lipid-derived radical production as sensitive to oxygen concentration in an inversely dependent manner.

Materials and methods

Materials

Soybean lipoxygenase-1 (type I-b), linoleic acid, 5-(dimethylamino)naphthalene-1-sulphonyl (dansyl) chloride, Chelex®100 and N-*tert*-butyl-α-phenylnitrone (PBN) were purchased from Sigma Aldrich-Japan (Tokyo, Japan). All other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan). 4-[5-(Dimethylamino)-1-naphthalenesulphonamido]-2,2,6,6-tetramethylpiperidine-1-oxyl (dansyl-TEMPO) was synthesized according to a previous report (structure, Figure 1B) [26]. Buffer solutions were treated three times for 10 min with gas displacement using a N_2 gas, 1% O_2/99% N_2 gas mixture or air in a sealed chamber (Fukuoka Oxygen Mfg. Co., Ltd., Fukuoka, Japan). After displacement, oxygen concentrations were measured using biological oxygen monitoring equipment (YSI/Nanotech Inc., Kanagawa, Japan), resulting in $0.8 \pm 0.2\%$, $1.7 \pm 0.1\%$ and $21.6 \pm 0.1\%$ oxygen content; all experiments were performed in the sealed chamber.

Animals

All procedures and animal care were approved by the Committee on Ethics of Animal Experiments, Faculty of Pharmaceutical Sciences, Kyushu University, and conducted according to the Guidelines for Animal Experiments of the Faculty of Pharmaceutical Sciences, Kyushu University. Male Wistar rats were purchased from Kyudo Co., Ltd. (Saga, Japan) and maintained in a temperature and humidity-controlled room with a standard diet *ad libitum* (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water.

Electron spin resonance measurements

Electron spin resonance (ESR) spectra were monitored using a X-band (9.45 GHz) ESR spectrometer (JES-FA100; JEOL, Tokyo, Japan) at room temperature, microwave power at 10 mW, field modulation at 100 kHz, modulation width at quarter to half line width of each compound and an external magnetic field range of 5 mT.

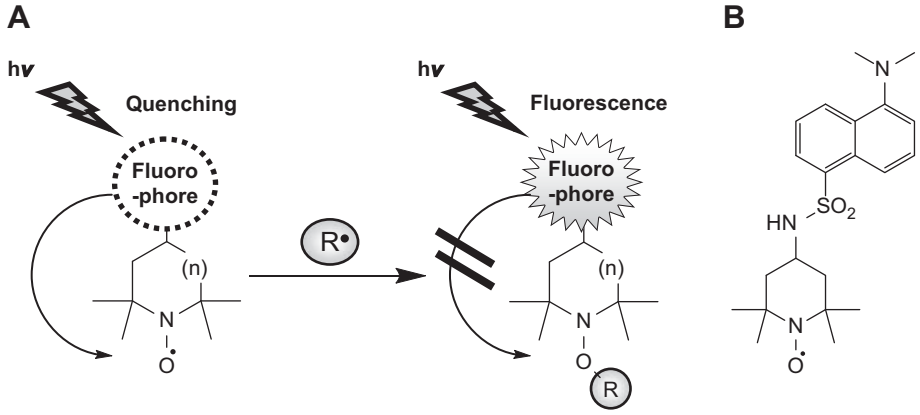

Figure 1. Concept of a spin-labelled fluorophore sensor for free radicals. By reacting with a free radical, fluorescence was recovered due to the loss of an unpaired electron of nitroxide (A). Chemical structure of dansyl-TEMPO (B).

Fluorescence experiments

Fluorescence emission spectra and steady-state fluorescence intensities were recorded on a Corona grading microplate reader (SH-9000Lab, Corona Electric Co., Ltd., Ibaraki, Japan). Fluorescence spectra of dansyl-TEMPO were measured at excitation and emission wavelengths of 335 and 487 nm, respectively.

Detection of lipid-derived radicals

Lipid-derived radicals generated in a linoleic acid/lipoxygenase system were trapped with dansyl-TEMPO or PBN as follows. A linoleic acid (0.5 mM) emulsion in 100 mM phosphate buffer (pH 7.4 and treated with Chelex®100) containing 2% ethanol was mixed with dansyl-TEMPO (250 μM) or PBN (200 mM) in phosphate buffer containing dimethyl sulphoxide and then soybean lipoxygenase-1 at 0.1 mg/mL in phosphate buffer added to the mixture. After incubation, the reaction was stopped with ice cold acetonitrile addition, the solution centrifuged at 10 000*g* for 5 min and the supernatant subjected to the methods described below.

Peroxidation of linoleic acid and microsomal suspension

Peroxidation of 0.5 mM linoleic acid was initiated by lipoxygenase-1 at 1.0 mg/mL in 1.5 mL tubes and, after 10 min, the degree of lipid peroxidation evaluated using a thiobarbituric acid reactive substances (TBARS) assay. Here, a tube containing 40 μL of 20% acetic acid, 60 μL of 1.3% (w/v) thiobarbituric acid dissolved in 0.3% NaOH and 15 μL of 10% (w/v) sodium dodecyl sulphate was gently shaken at 60°C for 40 min, cooled and the fluorescence measured at 512 and 553 nm for excitation and emission, respectively. Peroxidation of a microsomal suspension at 0.5 mg/mL was initiated by addition of cysteine (500 μM) and ferrous sulphate (10 μM). For this, a microsomal suspension was prepared according to a previous report [28]. Briefly, liver samples, obtained from male Wistar rats after euthanasia by deep anaesthesia, were washed and homogenized in chilled phosphate-buffered saline. The homogenates were centrifuged at 700 *g* for 10 min at 4°C and the supernatants centrifuged at 7000 *g* and 4°C for 10 min. The resulting supernatants were then centrifuged at 105 000 *g* and 4°C for 60 min and the samples incubated in 1.5 mL tubes at 37°C for 15 min. Lipid peroxidation was subsequently measured using the TBARS assay described above. A standard curve of 1,1,3,3-tetraethoxypropane was employed to quantitate the malondialdehyde produced. Microsomal protein concentrations were determined using a BCA protein assay kit (Thermo Scientific Pierce, Rockford, IL).

Liquid chromatography/high resolution mass spectroscopy

The liquid chromatography/high resolution mass spectroscopy (LC/HRMS) system consisted of an Agilent 1200 HPLC system (Agilent Technologies, Inc., CA) and microTOF II LC-MS system (Bruker Daltonics, Inc., MA) equipped with an electrospray ionization source. Chromatographic conditions for lipid-derived radical-nitroxide adduct quantitation were a TSKgel ODS-80Ts QA column (4.6 mm internal diameter × 15 cm), 0.1% formic acid containing 75% acetonitrile (v/v) eluent, 0.2 mL/min flow rate, 25°C column temperature and detection at 240 nm. The LC flow rate at 0.2 mL/min into the mass spectrometer inlet was adjusted via a splitter to 180 μL/min.

Oxygen consumption

Biological oxygen monitoring equipment, with an electrode calibrated by air-saturated water, was used to assess oxygen concentrations at 37°C. The sample chamber contained 0.5 mM cysteine, 0.5 mg/ml

microsomal suspension, 10 μM ferrous sulphate and 15 μM dansyl-TEMPO (added in DMSO with a final DMSO concentration of $< 0.3\%$).

Statistical analysis

Statistical analyses were carried out using an Excel statistics program (Microsoft Corporation, USA, 2008) and data analysed by an unpaired Student's *t*-test with the results expressed as the mean ± standard deviation and $p < 0.05$ considered statistically significant.

Results and discussion

Spectroscopic detection of the lipid-derived radicals

The change in the fluorescence and ESR signal intensities of dansyl-TEMPO were measured during reactions with lipid radicals derived from a linoleic acid/lipoxygenase system (Figures 2A and B). The fluorescence intensities were observed to increase as a function of time after lipoxygenase addition and, conversely, the ESR signal intensities decreased. The dansyl-TEMPO ESR signal was not restored by addition of the oxidizing species, potassium ferricyanide; the corresponding hydroxylamine reaction product was absent. When the reaction mixture was heated to 100°C for 5 min, the ESR signal intensities of dansyl-TEMPO reappeared to 75% of the intensity of a solution without lipoxygenase (data not shown). These results indicated that nitroxide reacted with carbon-centred radicals and mainly produced alkoxyamine derivatives. Alkoxyamines derived from radial–radical reactions are reported to be thermally unstable and dissociate into the corresponding nitroxide and alkyl radicals via a reversible homolysis process promoted by heating of the solution [29]. LC/HRMS was employed to reveal the reaction productions and showed three distinct peaks detected at 21 min (m/z; 700.4328), 29 min (m/z; 684.4398) and 37 min (m/z; 684.4398); these peaks disappeared after heating to 100°C (Figure S1). It has been reported that 13- and/or 9-linoleic acid and 12,13-epoxylinoleic acid radicals were produced in the linoleic acid/lipoxygenase system [30] and, based on this report, the m/z of 700.4328 was calculated for the protonated 12,13-epoxylinoleic acid radical-dansyl-TEMPO adduct ($C_{39}H_{62}N_3O_6S$ $[M + H]^+$; 700.4354) and m/z of 684.4398 for the protonated 13- or 9-linoleic acid radical-dansyl-TEMPO adducts ($C_{39}H_{62}N_3O_5S$ $[M + H]^+$; 684.4405). More detailed analysis of the reaction products is required for further elucidation, as several isomers could exist from this linoleic acid/lipoxygenase system and the products would be susceptible to secondary reactions, such as diol formation, dimerization and epoxy reactions. The fluorescence intensities were observed to increase in a lipoxygenase concentration-dependent manner (Figure 2C). These results suggested that dansyl-TEMPO reacted with lipid-based carbon-centred radicals.

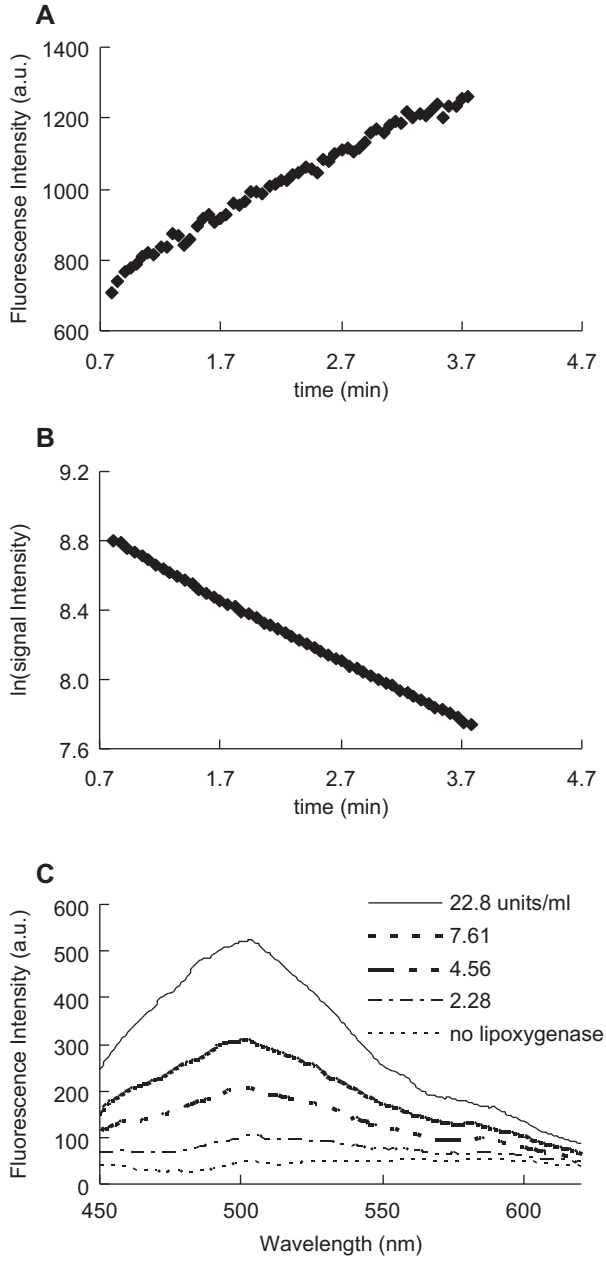

Figure 2. Time-dependent change of fluorescence (A) and ESR signal intensity (B) of dansyl-TEMPO (5 μM) induced by linoleic acid (0.5 mM) and lipoxygenase (0.1 mg/mL) system. (C) Fluorescence emission spectra of dansyl-TMEPO reacted with lipid-derived radicals produced by the linoleic acid/lipoxygenase system, excitation at 335 nm.

Oxygen concentration dependent lipid-derived radical generation: Spin-trapping technique

The effect of oxygen concentration on lipid-derived radical production was evaluated using the spin trapping agent PBN, which traps free radicals and forms paramagnetic spin adducts. In principle, PBN can

trap any of the lipid-derived carbon centred radical, alkoxy radical and peroxy radical [31]. However, the short half-life of PBN adducts with oxygen-centred radical restricts its use to the detection of carbon-centred radical adducts only [32]. Here, the ESR signal intensities of PBN adducts decreased with increased oxygen concentration (Figure 3). This indicated that oxygen changes directly affected lipid radical-chain reactions, resulting in decreased amounts of PBN adducts. However, oxygen must be involved in the enzymatic reaction mechanism of lipoxygenase [33]. The hydrogen at carbon atom C-11 of linoleic acid is transferred to the Fe(III)-OH co-factor on the enzyme, forming a radical intermediate substrate and Fe(II)-OH_2, and the subsequent reaction with molecular oxygen eventually leads to hydroperoxyoctadecadienoic acid and the regeneration of Fe(III)-OH. Therefore, under low oxygen conditions, the reaction of the linoleic acid/lipoxygenase system might not have proceeded well due to most enzyme remaining as Fe(II)-OH_2 forms, although ESR signal intensities at lower oxygen concentrations were higher than in higher oxygen.

Oxygen concentration dependent lipid-derived radical generation: Lipoxygenase system

The lipid peroxidation products under different oxygen conditions, evaluated by the TBARS assay and dansyl-TEMPO fluorescence method (Figure 4) showed that, as the oxygen concentration in solution increased, the TBARS concentrations increased. Conversely, the dansyl-TEMPO fluorescence intensity decreased with increasing oxygen. Under low oxygen, the reaction efficacy between the carbon-centred radicals and O_2 might have been decreased, resulting in more dansyl-TEMPO reacting with carbon-centred radicals. Furthermore, analyses of reaction products by LC/MS revealed that the amounts of the unreacted linoleic acid were larger under lower oxygen (data not shown). The absolute amounts of lipid-derived carbon-centred radicals under lower oxygen would have been fairly low in the linoleic acid/lipoxygenase system, but the dansyl-TEMPO fluorescence was significantly increased. Although the reaction rate of carbon-centred radicals with O_2 have been reported to be higher ($\sim 2\text{–}4 \times 10^9\ M^{-1}s^{-1}$) [34] than with commercially available nitroxides ($\sim 1\text{–}8 \times 10^8\ M^{-1}s^{-1}$) [35], dansyl-TEMPO appeared to be sensitive in monitoring carbon-centred radical production, especially under low oxygen.

Oxygen concentration dependent lipid-derived radical generation: Microsomes

To avoid the effect of oxygen in these enzymatic reactions, rat liver microsomes were utilized as a biomembrane model system, which here showed that TBARS concentration increased with oxygen concentration in a concentration-dependent manner and, conversely, the fluorescence intensities decreased, similar to the results from the linoleic acid/lipoxygenase system (Figure 5). These results demonstrated that dansyl-TEMPO could be used to detect lipid-derived radicals not only with an isolated lipid, but also from a biomembrane system. Based on Le Chatelier's principle,

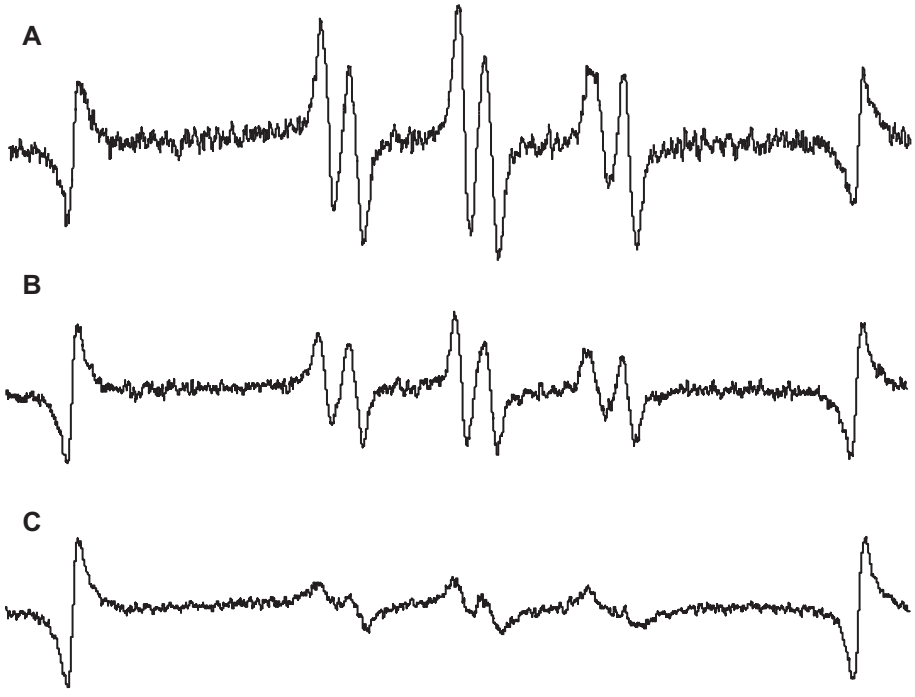

Figure 3. ESR spectrum trapped with PBN. Oxygen concentration of A, B and C, 0.8%, 1.7% and 21.6%, respectively; carbon-centred radicals produced by linoleic acid/lipoxygenase system.

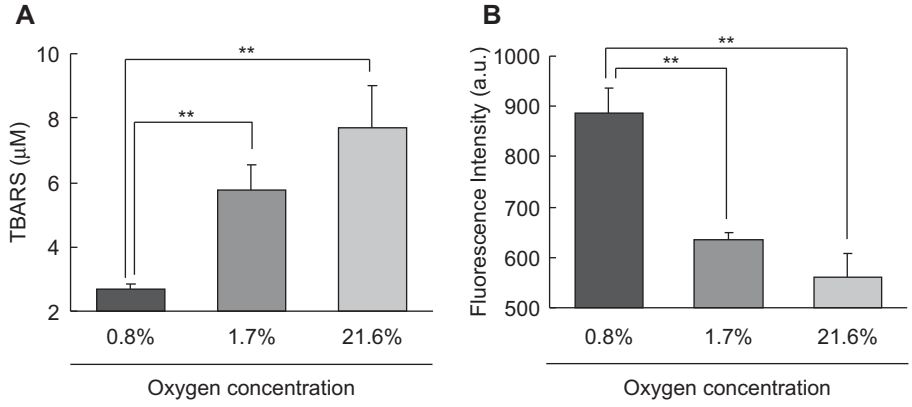

Figure 4. TBARS concentration from linoleic acid peroxidation induced by the linoleic acid and lipoxygenase system (A). Fluorescence intensities of dansyl-TEMPO reacted with the linoleic acid/lipoxygenase system (B). Values, means ± standard deviation; $n = 3$; $^{**}p < 0.005$.

the lipid peroxidation equilibrium reaction would have been shifted by the oxygen concentration and, as a result, the TBARS concentration was altered by oxygen in a concentration-dependent manner. Dansyl-TEMPO addition inhibited TBARS production to that of the control even under higher oxygen (data not shown). Furthermore, ESR signal intensities from PBN spin adducts decreased under higher oxygen and, under these conditions, the effect of dansyl-TEMPO on oxygen consumption was examined during lipid peroxidation. After addition of cysteine and ferrous sulphate to microsomal solutions, oxygen consumption was initiated. Interestingly, addition of dansyl-TEMPO increased the slope of the line compared with vehicle; dansyl-TEMPO attenuated oxygen consumption in this system (Figure S2). Therefore, it is indicated that dansyl-TEMPO could have reacted with lipid-derived radicals in the microsomes and is thus a good candidate for a detection agent for lipid-derived radicals.

In conclusion, it was revealed the pro-fluorescent nitroxide dansyl-TEMPO acted as a sensitive detector of lipid-derived carbon-centred radicals through its ESR and fluorescence spectra responses for the first time (Figure 6). Under hypoxic conditions, lipid peroxidation decreased although lipid-derived radicals trapped with dansyl-TEMPO increased. Furthermore, dansyl-TEMPO addition inhibited the production of radicals from lipid peroxidation and attenuated oxygen consumption during the lipid peroxidation chain reaction in the microsomal solution, due to the competition reaction between the oxygen molecule and dansyl-TEMPO in lipid peroxidation; here the concentration of 1% oxygen in solution was ~13.17 μM and dansyl-TEMPO 15 μM. The reaction rate constant for carbon-centred radicals with O_2 is higher (~$2–4 \times 10^9$ $M^{-1}s^{-1}$) [34] than with the commercially available nitroxides (~$1–8 \times 10^8$ $M^{-1}s^{-1}$) [35]. Although the rate constants for dansyl-TEMPO and oxygen molecules was not known in the present linoleic acid/lipoxygenase system or the microsomes, dansyl-TEMPO reacted significantly with lipid-derived radicals. The oxygen tension in cancer or tumour regions has been reported to be <10 mmHg [36] (~17.33 μM)

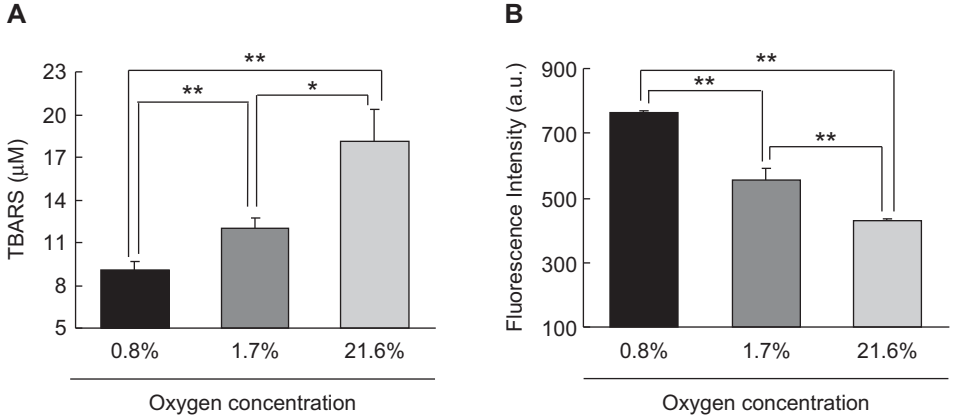

Figure 5. TBARS concentration in microsomal suspension peroxidation induced by cysteine (0.5 mM) and $FeSO_4$ (10 μM) in a 0.5 mg/mL microsomal suspension (A). Fluorescence intensities of dansyl-TEMPO reacted with microsome peroxidation (B). Values, means ± standard deviation; $n = 3$; $^{*}p < 0.05$; $^{**}p < 0.005$.

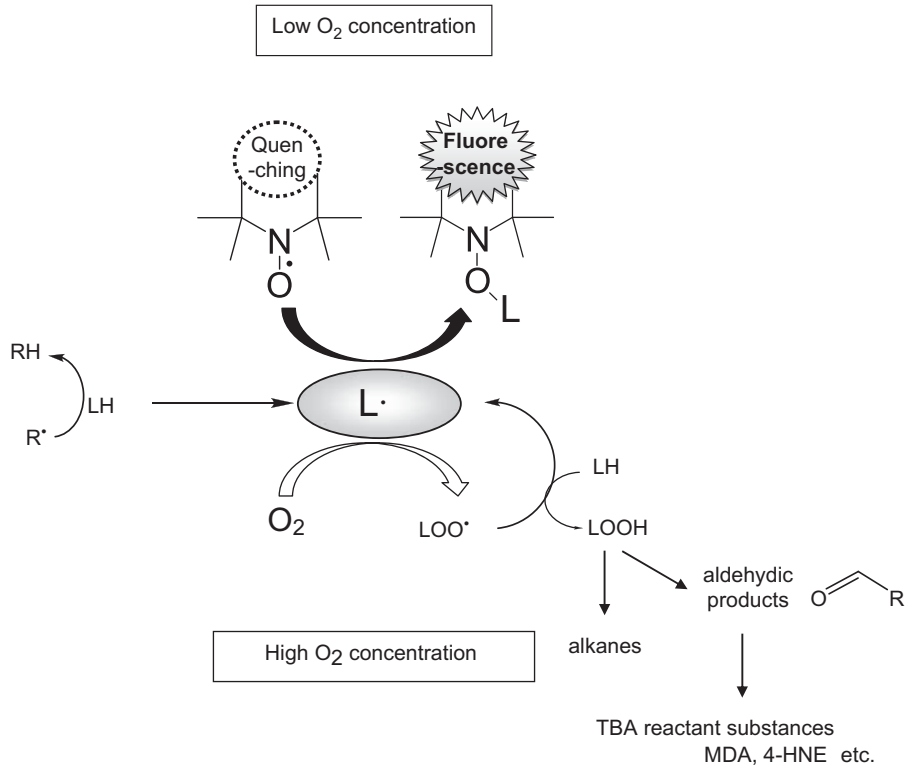

Figure 6. Schematic of the dansyl-TEMPO fluorescence emission system. In this mechanism, dansyl-TEMPO competes with the oxygen molecule and, thus, under hypoxic conditions, lipid-derived radials trapped with dansyl-TEMPO increased and fluorescence intensity increased. Then, dansyl-TEMPO addition inhibited lipid peroxidation and attenuated oxygen consumption during the lipid peroxidation chain reaction in the microsomal solution.

and, thus, such hypoxic regions might have less lipid peroxidation and increased lipid-derived radicals compared with normoxic regions. Substances with reactivity for lipid-derived radicals, such as dansyl-TEMPO and nitroxides, have the potential to inhibit the rate of lipid peroxidation by binding to lipid-derived radicals and thus attenuating oxygen consumption at the region of interest. Increased free radicals may induce hypoxia followed by acceleration of mitochondria-dependent apoptosis [37] and the inhibition of a chain reaction leading to lipid-derived radicals might have an advantage by inhibiting gene expression induced by hypoxia. This hypothesis can be clarified through the further cell and animal experiments. Furthermore, this pro-fluorescent nitroxide can serve as a sensitive detector for carbon-centred radicals. Recently here, the nitroxide functions such as reactivity and *in vivo* stability were successfully regulated by a new synthetic method [28,38,39] and this new series of nitroxide compounds combined with fluorescence could be useful in detecting not only free radicals but also in regulating the redox environment by regulating reactivity based on redox potential. This fluorescent nitroxide switching method, through the interaction between an unpaired electron and a fluorophore, will provide valuable information regarding free radicals during oxidative stress.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the contest and writing of the paper.

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Supplementary material available online

Supplementary Figures S1 and S2