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Identification of a radical formed in the reaction mixtures of oxidized phosphatidylcholine with ferrous ions using HPLC–ESR and HPLC–ESR–MS

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Abstract

Identification of free radicals was performed for the reaction mixtures of autoxidized 1,2-dilinoleoylphosphatidylcholine (DLPC) with ferrous ions (or DLPC hydroperoxide with ferrous ions) and of DLPC with soybean lipoxygenase using electron spin resonance (ESR), high performance liquid chromatography (HPLC)–ESR and HPLC–ESR–mass spectrometry (MS) combined use of spin trapping technique. ESR measurements of the reaction mixtures showed prominent signals with hyperfine coupling constants ($a^{N} = 1.58$ mT and $a^{H}\beta = 0.26$ mT). Outstanding peaks with almost same retention times (autoxidized DLPC, 36.9 min; DLPC hydroperoxide, 35.0 min; DLPC with soybean lipoxygenase, 37.1 min) were observed on the elution profile of the HPLC–ESR analyses of the reaction mixtures. HPLC–ESR–MS analyses of the reaction mixtures gave two ions at *m/z* 266 and 179, suggesting that 4-POBN/pentyl radical adduct forms in these reaction mixtures.

Keywords: *Lipid peroxidation, HPLC–ESR, HPLC–ESR–MS, pentyl radical, spin trapping*

Introduction

Considerable evidence indicates an important role of reactive free radicals derived primarily from lipids and oxygen molecules in the pathophysiology of a wide spectrum of disorder including atherosclerosis, ischemia-reperfusion injury, inflammatory disease, cancer and aging [1]. Lipid peroxidation in cell membrane phospholipids induced by reactive oxygen species (ROS) and/or free radicals leads to membrane damage and has been proposed to be a major mechanism for the onset of several pathological events *in vivo* [2]. In addition to phosphatidylcholine hydroperoxide [3], aldehydes, volatile hydrocarbons [4,5], phosphatidylcholines with a short *sn*-2 acyl residues [6–9] and free radicals [10,11] have been detected from phosphatidylcholines under various kinds of oxygen stress. To our knowledge, free radicals derived from phosphatidylcholines have not been, however, identified using high performance liquid chromatography–electron spin resonance–mass spectrometry (HPLC–ESR–MS).

Free radicals have been successfully detected using ESR spectroscopy combined with spin trapping

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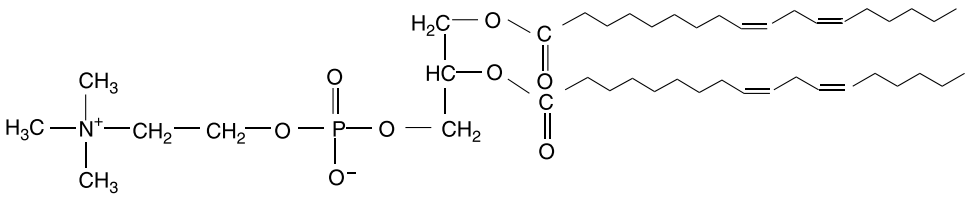


Figure 1. Structure of 1,2-dilinoleoylphosphatidylcholine.

technique [12,13]. HPLC–ESR [14–16] and HPLC–ESR–MS [17] have also been employed to detect and identify the radical adducts. Based on mass spectral data, several radical species such as pentyl radical, 7-carboxyheptyl radical, 1-(7-carboxyheptyl)-4,5-epoxy-2-decenyl radical and 1-(7-carboxyheptyl)-4,5-dihydroxy-2-decenyl radical were identified in the reaction mixtures of linoleic acid with soybean lipoxygenase (or 13-hydroperoxy-9,11-octadecadienoic acid or peroxidized linoleic acid with ferrous ions) [18–26].

To clarify the mechanism of the peroxidation of phosphatidylcholines, it is essential to determine the structures of the radical species. In this paper, pentyl radical is detected and identified in the reaction mixtures of autoxidized 1,2-dilinoleylphosphatidylcholine (DLPC) with ferrous ions (Figure 1) (or DLPC hydroperoxide with ferrous ions) and of DLPC with soybean lipoxygenase using ESR, HPLC–ESR and HPLC–ESR–MS combined use of spin trapping technique. Furthermore, a possible reaction path is brought up for the formation of the pentyl radical.

Materials and methods

Materials

α-(4-Pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN) was purchased from Tokyo Kasei Kogyo, Ltd. (Tokyo, Japan). Soybean lipoxygenase (Type V) and DLPC were from Sigma-Aldrich Co. (St. Louis, MO, USA). Ferrous ammonium sulfate was obtained from Kishida Chem. Co. (Osaka, Japan). Ethylenediaminetetraacetic acid disodium salt (EDTA) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals used were of analytical grade.

Preparation of autoxidized DLPC

DLPC was oxidized by bubbling oxygen gas through a 5 mg/20 ml H_2O solution of 1,2-dilinoleoylphosphatidylcholine at 70°C for 5 h.

The reaction mixture of autoxidized DLPC with ferrous ions

The reaction mixture contained 50 μg/ml oxidized DLPC (or 50 μg/ml DLPC), 0.1 M 4-POBN, 1.7 mM $FeSO_4(NH_4)_2SO_4$, 1.7 mM EDTA, and 18 mM phosphate buffer (pH 7.4). The reaction was started by adding $FeSO_4(NH_4)_2SO_4$. After 30 min reaction at 25°C, the reaction mixtures were applied to ESR, HPLC–ESR and HPLC–ESR–MS.

The reaction mixture of DLPC with soybean lipoxygenase

The complete reaction mixture contained 94 μg/ml DLPC, 158,000 units/ml soybean lipoxygenase, 94 mM 4-POBN, 9.4 mM sodium cholate and 19 mM borate buffer (pH 9.0). The reaction was started by adding soybean lipoxygenase. After 27 min reaction at 25°C, the reaction mixtures were applied to ESR, HPLC–ESR and HPLC–ESR–MS.

Preparation of the DLPC hydroperoxide

DLPC was oxidized by soybean lipoxygenase. The reaction mixture contained 96.8 μg/ml DLPC, 814,000 units/ml soybean lipoxygenase, 9.7 mM sodium cholate and 19.4 mM borate buffer (pH9.0). The reaction was performed for 20 min at 20°C. According to the method of Crawford et al. [27] DLPC hydroperoxide fraction was obtained. The fraction consisted of two hydroperoxides. One of the products contained an oxidized and an unoxidized fatty acid. In the other product, both fatty acids were oxidized. The concentration of the DLPC hydroperoxide was determined from its absorbance at 234 nm ($\epsilon = 25{,}600\ cm^{-1}\,M^{-1}$) [28].

The reaction mixture of ferrous ion with the DLPC hydroperoxide

The complete reaction mixture contained 100 mM 4-POBN, 9.4 mM sodium cholate, 43 mM phosphate buffer (pH 7.4), 83 μM DLPC hydroperoxide and 0.33 mM $FeSO_4(NH_4)_2SO_4$. The reaction was started by adding $FeSO_4(NH_4)_2SO_4$. The reaction was performed at 25°C for 2 min. The reaction mixture was applied to ESR, HPLC–ESR and HPLC–ESR–MS.

ESR measurements

The ESR spectra were obtained using a model JES-FR30 Free Radical Monitor (JEOL Ltd., Tokyo, Japan). Aqueous samples were aspirated into a Teflon tube centered in a microwave cavity. Operating conditions of the ESR spectrometer were: power, 4 mW; modulation width, 0.1 mT; center of magnetic field, 337.000 mT; sweep time, 4 min; sweep width,

10 mT; time constant, 0.3 s. Magnetic fields were calculated by the splitting of MnO (δH3-4 = 8.69 mT).

HPLC–ESR chromatography

An HPLC used in the HPLC–ESR consisted of a model 7125 injector (Reodyne, Cotati, CA, USA), a model 655A-11 pump with a model L-5000 LC controller (Hitachi Ltd., Ibaragi, Japan). A semi-preparative column (300 mm long × 10 mm i.d.) packed with TSKgel ODS-120T (TOSOH Co., Tokyo, Japan) was used. Flow rate was 2.0 ml/min throughout the HPLC–ESR experiments. For the HPLC–ESR, two solvents were used: solvent A, 50 mM acetic acid; solvent B, 50 mM acetic acid/-acetonitrile (20:80, v/v). A following combination of isocratic and linear gradient was used: 0–40 min, 100–0% A (linear gradient); 40–70 min, 100% B (isocratic). The eluent was introduced into a model JES-FR30 Free Radical Monitor (JEOL Ltd., Tokyo, Japan). The ESR spectrometer was connected to the HPLC with a Teflon tube, which passed through the center of the ESR cavity. The operating conditions of the ESR spectrometer were: power, 4 mW; modulation width, 0.2 mT; time constant, 1 s. The magnetic field was fixed at the third peak in the doublet-triplet ESR spectrum ($a^N = 1.58$ mT and $a^H\beta = 0.26$ mT) of the 4-POBN radical adduct (Figures 2, 5 and 7).

HPLC–ESR–MS chromatography

HPLC and ESR conditions were as described in the HPLC–ESR. The operating conditions of the mass spectrometer were: nebulizer, 180°C; aperture 1, 120°C; N_2 controller pressure, 2.0 kg/cm^2; drift voltage, 70 V; multiplier voltage, 1800 V; needle voltage, 3000 V; polarity, positive; resolution, 48.

The mass spectra were obtained by introducing the eluent from the ESR detector into the LC-MS system just before the peak were eluted. The flow rate was kept at 50 μl/min while the eluent was introducing into the mass spectrometer.

Results and discussion

ESR measurements of the reaction mixtures of autoxidized DLPC with ferrous ions

ESR spectrum of the complete reaction mixture of autoxidized DLPC with ferrous ions was measured (Figure 2A). A prominent ESR spectrum ($a^N = 1.58$ mT and $a^H\beta = 0.26$ mT) was observed in the complete reaction mixture. The ESR spectrum was hardly observed for the complete reaction mixture without ferrous ions (or autoxidized DLPC) (Figure 2C and D). For the complete reaction mixture of unoxidized DLPC, a weak ESR spectrum was observed (Figure 2B). In the absence of EDTA, the intensity of the ESR signal decreased to 53% of the complete reaction mixture (Figure 2E).

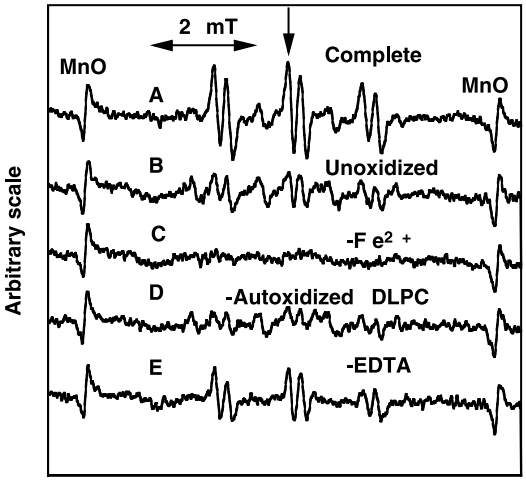


Figure 2. ESR spectra of the reaction mixtures of autoxidized DLPC with ferrous ions. The reaction and ESR conditions were as described under "Materials and methods". (A) a complete reaction mixture of autoxidized DLPC with ferrous ions; (B) a complete reaction mixture of unautoxidized DLPC with ferrous ions; (C) same as in A except that ferrous ions were omitted; (D) same as in A except that autoxidized DLPC was omitted; (E) same as in A except that EDTA was omitted.

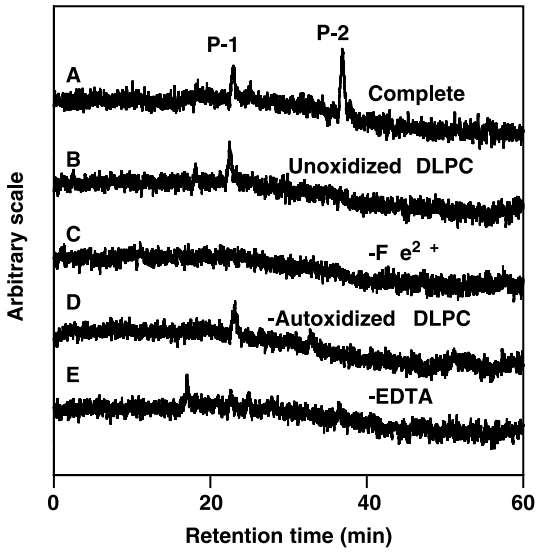


Figure 3. HPLC-ESR analyses of reaction mixtures of autoxidized DLPC with ferrous ions. The reaction and HPLC-ESR conditions were as described under "Materials and methods". Total volume of the reaction mixtures was 3.0 ml. (A) a complete reaction mixture of autoxidized PCDL with ferrous ions; (B) a complete reaction mixture of unoxidized DLPC with ferrous ions; (C) same as in A except that ferrous ions were omitted; (D) same as in A except that autoxidized DLPC was omitted; (E) same as in A except that EDTA was omitted. Retention times of peak 1(P-1) and peak 2 (P-2) were 23.0 and 36.9 min, respectively.

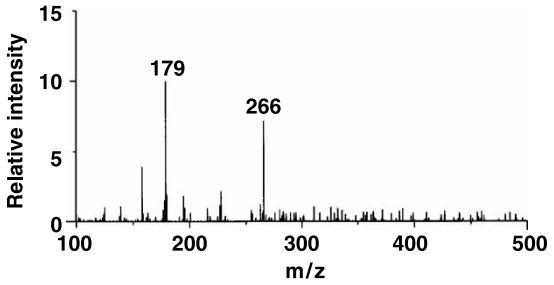


Figure 4. HPLC-ESR-MS analysis of the reaction mixture of autoxidized DLPC with ferrous ions. The reaction and HPLC-ESR-MS conditions were as described under "Materials and methods". Total volume of the reaction mixture was 5.0 ml.

HPLC–ESR and HPLC–ESR–MS analyses of the reaction mixture of autoxidized DLPC with ferrous ions

To identify the radicals formed in the complete reaction mixture of autoxidized DLPC with ferrous ions, HPLC–ESR analyses were performed. Two prominent peaks (peaks 1 and 2) were separated on the HPLC–ESR elution profile of the complete reaction mixture of autoxidized DLPC with ferrous ions (Figure 3A). The retention times of the peaks 1 and 2 are 23.0 and 36.9 min, respectively. The peak 1 was observed for the complete reaction mixture without autoxidized DLPC, suggesting that the peak 1 radical is not derived from autoxidized DLPC (Figure 3D). The peak 2 was not observed for the complete reaction mixture in the absence of ferrous ions (or EDTA) (Figure 3C and E). For the complete reaction mixture of unoxidized DLPC, the peak 2 was not observed (Figure 3B). Thus, the peak 2 could be derived from the autoxidized DLPC.

To determine the structures of the peak 2, HPLC–ESR–MS analysis was performed. HPLC–ESR–MS analysis of the peak 2 compound gave ions at *m/z* 266 and 179 (Figure 4). The ion *m/z* 266 corresponds to the protonated molecular ion of 4-POBN/pentyl radical adduct, $(M + H)^+$. A fragment ion at *m/z* 179 corresponds to the loss of $[(CH_3)_3C(O)N]$ from the protonated molecular ion.

ESR measurements of the reaction mixtures of DLPC with soybean lipoxygenase

ESR spectrum of the complete reaction mixture of DLPC with soybean lipoxygenase was measured (Figure 5A). A prominent ESR spectrum ($a^N = 1.58$ mT and $a^H\beta = 0.26$ mT) was observed in the complete reaction mixture. The ESR spectrum was hardly observed for the complete reaction mixture without sodium cholate (or DLPC or soybean lipoxygenase; Figure 5B–D), suggesting that cholate is essential for the reaction of DLPC with soybean lipoxygenase. Eskola et al. have shown that cholate is required for the oxygenation of polyunsaturated phosphatidylcholine by soybean lipoxygenase [29].

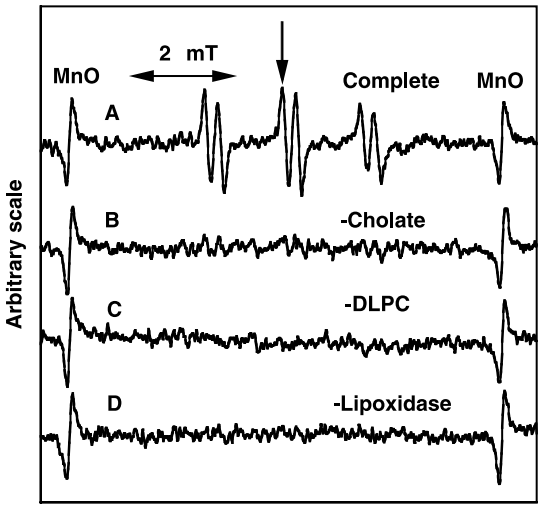


Figure 5. ESR spectra of the reaction mixtures of DLPC with soybean lipoxygenase. The reaction and ESR conditions were as described under "Materials and methods". (A) a complete reaction mixture of DLPC with soybean lipoxygenase; (B) same as in A except that sodium cholate was omitted; (C) same as in A except that DLPC was omitted; (D) same as in A except that soybean lipoxygenase was omitted.

HPLC–ESR and HPLC–ESR–MS analyses of the reaction mixture of DLPC with soybean lipoxygenase

To identify the radicals formed in the complete reaction mixture of DLPC with soybean lipoxygenase, HPLC–ESR analyses were performed (Figure 6A). A peak was observed on the HPLC–ESR elution profile of the complete reaction mixture of DLPC with

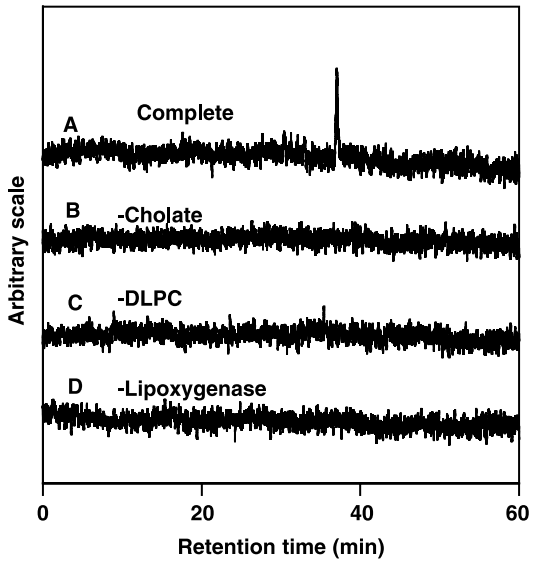


Figure 6. HPLC-ESR analyses of reaction mixtures of DLPC with soybean lipoxygenase. The reaction and HPLC-ESR conditions were as described under "Materials and methods". Total volume of the reaction mixture was 3.2 ml. (A) a complete reaction mixture of DLPC with soybean lipoxygenase; (B) same as in A except that sodium cholate was omitted; (C) same as in A except that DLPC was omitted; (D) same as in A except that soybean lipoxygenase was omitted.

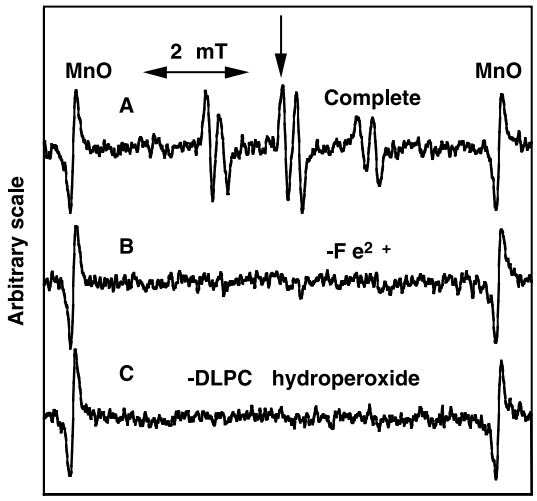


Figure 7. ESR analyses of the reaction mixtures of DLPC hydroperoxide with ferrous ions. The reaction and ESR conditions were as described under "Materials and methods". (A) a complete reaction mixture of DLPC hydroperoxide with ferrous ions; (B) same as in A except that ferrous ions were omitted; (C) same as in A except that DLPC hydroperoxide was omitted.

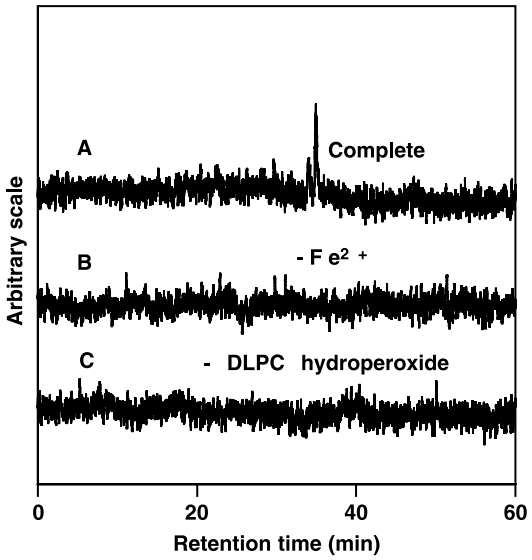


Figure 8. HPLC-ESR analyses of reaction mixtures of DLPC hydroperoxide with ferrous ions. The reaction and HPLC-ESR conditions were as described under "Materials and methods". Total volume of the reaction mixture was 3.0 ml. (A) a complete reaction mixture of DLPC hydroperoxide with ferrous ions; (B) same as in A except that ferrous ions were omitted; (C) same as in A except that DLPC hydroperoxide was omitted.

soybean lipoxygenase. The retention time of the peak is 37.1 min. The peak was not observed for the complete reaction mixture without sodium cholate (or DLPC or soybean lipoxygenase).

To determine the structures of the peak, HPLC–ESR–MS analysis was performed. HPLC–ESR–MS analysis of the peak compound gave ions at *m*/*z* 266 and 179 (data not shown). This result indicates the formation of pentyl radicals in this reaction mixture as described in the reaction of autoxidized DLPC with ferrous ions.

ESR measurements of the reaction mixtures of DLPC hydroperoxide with ferrous ions

To know whether or not DLPC hydroperoxide participates in the formation of the radicals, ESR spectrum of the complete reaction mixture of DLPC hydroperoxide with ferrous ions was measured (Figure 7A). A prominent ESR spectrum ($a^{N} = 1.58$ mT and $a^{H}\beta = 0.26$ mT) was observed in the complete reaction mixture. The ESR spectrum was hardly observed for the complete reaction mixture without ferrous ions (or DLPC hydroperoxide) (Figure 7B and C).

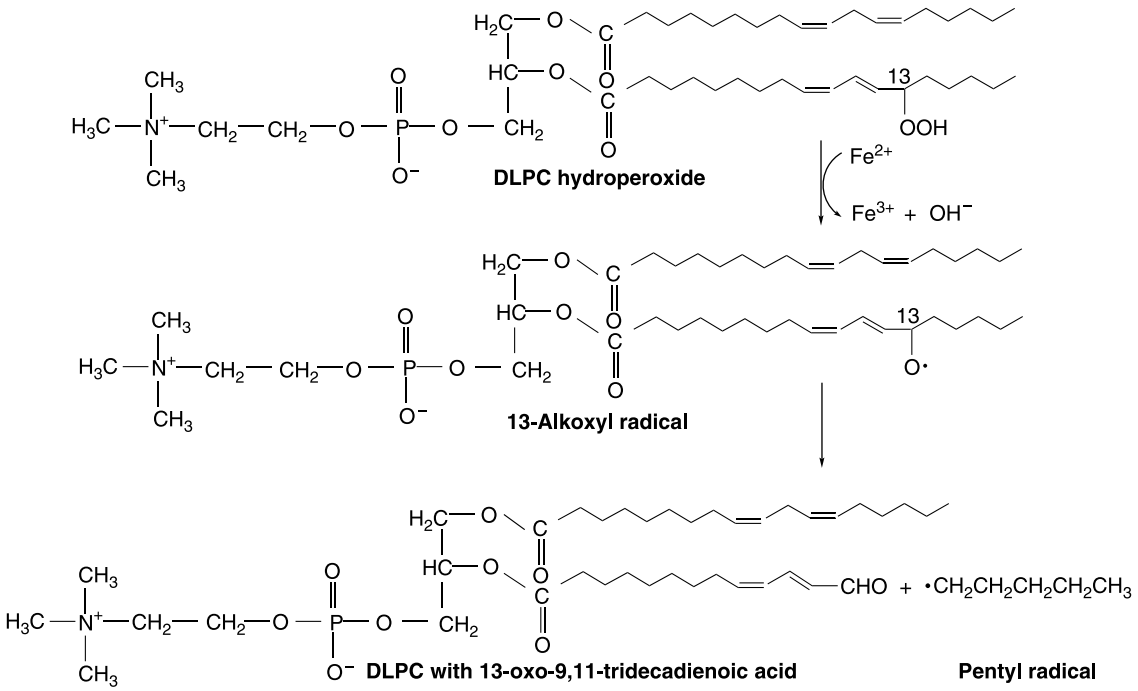
HPLC–ESR and HPLC–ESR–MS analyses of the reaction mixtures of DLPC hydroperoxide with ferrous ions

To identify the radicals formed in the complete reaction mixture of DLPC hydroperoxide with ferrous ions, HPLC–ESR analyses were performed (Figure 8A). A peak was observed on the HPLC–ESR elution profile of the complete reaction mixture of DLPC hydroperoxide with ferrous ions. The retention time of the peak is 35.0 min. The peak was not observed for the complete reaction mixture without ferrous ions (or DLPC hydroperoxide).

To determine the structures of the peak, HPLC–ESR–MS analysis was performed. HPLC–ESR–MS analysis of the peak gave ions at *m*/*z* 266 and 179 (data not shown). This result indicates the formation of pentyl radicals in this reaction mixture as described in the reaction of autoxidized DLPC with ferrous ions.

A Possible reaction path for the formation of the pentyl radical

In this paper, pentyl radical was detected and identified in the reaction mixtures of the autoxidized DLPC (or DLPC hydroperoxide) with ferrous ions and of DLPC with soybean lipoxygenase using ESR, HPLC–ESR and HPLC–ESR–MS combined use of spin trapping technique. A possible reaction path for the formation of the pentyl radicals is shown in Scheme 1. DLPC hydroperoxide with 13-hydroperoxylinoleic acid residue would generate in the process of autoxidation of DLPC (or hydroperoxidation of DLPC by soybean lipoxygenase). 13-Alkoxyl radical may possibly form through the reaction of DLPC hydroperoxide with 13-hydroperoxylinoleic acid residue with ferrous ions. The β scission of the 13-alkoxyl radical residue will result in the formation of pentyl radical [18,20,30]. Phosphatidylcholines with a short *sn*-2 acyl residues (13-oxo-9,11-tridecadienoic acid) simultaneously form through the β scission (Scheme 1). Indeed, it has been reported that the phosphatidylcholines with a short *sn*-2 acyl residues



Scheme 1. A possible reaction path for the formation of the pentyl radical.

form under oxygen stress [6–9]. Thus, this paper showed that the phosphatidylcholines with a short *sn*-2 acyl residues form through a radical reaction.

Some radicals can not be detected using spin trapping technique because of the instability of the radical adducts and of the difficulty in trapping the radicals. Therefore, some radicals other than pentyl radicals may form in the reaction mixtures of the autoxidized DLPC (or DLPC hydroperoxide) with ferrous ions and of DLPC with soybean lipoxygenase.

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