methods

Quantification of lipid alkyl radicals trapped with nitroxyl radical via HPLC with postcolumn thermal decomposition

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Abstract **Lipid alkyl radicals generated from polyunsaturated fatty acids via chemical or enzymatic H-abstraction have been a pathologically important target to quantify. In the present study, we established a novel method for the quantification of lipid alkyl radicals via nitroxyl radical spin-trapping. These labile lipid alkyl radicals were converted into nitroxyl radical-lipid alkyl radical adducts using 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-*N*-oxyl (CmΔP) (a partition coefficient between octanol and water is approximately 3) as a spin-trapping agent. The resulting CmΔP-lipid alkyl radical adducts were determined by HPLC with postcolumn online thermal decomposition, in which the adducts were degraded into nitroxyl radicals by heating at 100°C for 2 min. The resulting nitroxyl radicals were selectively and sensitively detected by electrochemical detection. With the present method, we, for the first time, determined the lipid alkyl radicals generated from linoleic acid, linolenic acid, and arachidonic acid via soybean lipoxygenase-1 or the radical initiator 2,2′-azobis(2,4-dimethyl-valeronitrile).**—Koshiishi, I., K. Tsuchida, T. Takajo, and M. Komatsu. **Quantification of lipid alkyl radicals trapped with nitroxyl radical via HPLC with postcolumn thermal decomposition.** ***J. Lipid Res.*** **2005.** 46: **2506–2513.**

Supplementary key words spin-trapping • high-performance liquid chromatography • lipoxygenases

Peroxidation of polyunsaturated fatty acids, which are components of cellular membrane and lipoproteins, leads to vital damage to several types of cells. Reactive oxygen/nitrogen species (1), such as hydroxyl radical, hypochlorous acid (2–4), peroxynitrite (5, 6), and singlet oxygen molecule (7–9) are involved in the peroxidation of polyunsaturated fatty acids in the inflammatory lesions. On the other hand, lipoxygenases are speculated to be involved in the peroxidation of polyunsaturated fatty acids in the atheromatous plaque (10). Commonly, lipid alkyl radicals seem to be necessarily produced as an intermediate in these reactions.

To detect labile free radicals via ESR, spin-trapping techniques have been established, in which unstable radicals react with spin-trapping agents to form relatively stable radical adducts (11). To date, nitrone spin-trapping agents, including α-[4-pyridyl-1-oxide]*N*-*tert*-butyl nitrone and 5,5′-dimethyl-1-pyroline-*N*-oxide, have been used to detect these lipid-derived radicals (12–17). Furthermore, Mason and coworkers (13–15) established an HPLC/ESR system to identify the resulting radical adducts. However, the trapping efficiency of these nitrone compounds toward lipid alkyl radicals is comparatively low. Consequently, these spin-trapping agents are not applicable to the quantification of lipid alkyl radicals.

Five- or six-membered cyclic nitroxyl radicals are relatively stable. Nitroxyl radicals appeared to possess an ability to scavenge carbon-centered radicals (18). This special character of nitroxyl radicals makes it possible to quantitatively trap carbon-centered radicals. Johnson, Caron, and Blough (19) used hydrophilic 3-aminomethyl-2,2,5,5-tetramethylpyrrolidine-*N*-oxyl to evaluate carbon-centered radical generation: in this method, the adducts were fluorometrically detected after derivatization of amino groups by fluorescamine.

The nitroxyl radical-carbon-centered radical adducts are generally labile at higher temperature, yielding corresponding nitroxyl radicals (20). Therefore, the resulting nitroxyl radicals should become a reporter substance. In the present study, we established a method for the determination of nitroxyl radical-lipid-derived carbon-centered radical adducts by the combination of HPLC and thermal decomposition.

EXPERIMENTAL PROCEDURES

Materials

Soybean lipoxygenase-1 (type I-b), linoleic acid, linolenic acid, arachidonic acid, and linolelaidic acid were purchased from

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Abbreviations: AMVN, 2,2′-azobis(2,4-dimethyl-valeronitrile); CmΔP, 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-*N*-oxyl; ECD, electrochemical detection; epoxy-$C_{18:2}$, linoleate epoxyallyl radical; LC/MS/MS, liquid chromatography-tandem mass spectrometry.

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Sigma. 2,2′-Azobis(2,4-dimethyl-valeronitrile) (AMVN) was obtained from Wako Pure Chemicals (Osaka, Japan). 3-Carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-N-oxyl (CmΔP) was purchased from Aldrich Chemical Co., Inc. The nitroxyl radicals were recrystallized in ethanol before use. TSKgel ODS-80Ts QA and TSKguardgel ODS-80Ts were purchased from Tosoh Co. (Tokyo, Japan). Chelex® 100 Resin (100–200 mesh) was purchased from Bio-Rad Laboratories. All other chemicals were of reagent grade.

Lipid-derived radical trapping by nitroxyl radicals

Lipid-derived radicals generated in the polyunsaturated fatty acid/lipoxygenase system were trapped with nitroxyl radicals as follows. Twenty microliters of 2 mM polyunsaturated fatty acid emulsion in 0.1 M phosphate buffer (pH 7.4; treated with Chelex 100) containing 2% ethanol was mixed with 10 μl of 4 mM nitroxyl radical in 0.1 M phosphate buffer (pH 7.4; treated with Chelex 100) and 10 μl of 4 μM soybean lipoxygenase-1 in 0.1 M phosphate buffer (pH 7.4; treated with Chelex 100) in a glass vial tube with screw cap (inner volume, 0.5 ml), and the solution was left to stand at room temperature (25–28°C) for 10 min. The reaction solution was mixed with 160 μl of cold acetonitrile, and then the solution was centrifuged at 10,000 *g* for 5 min. The supernatant was subjected to HPLC.

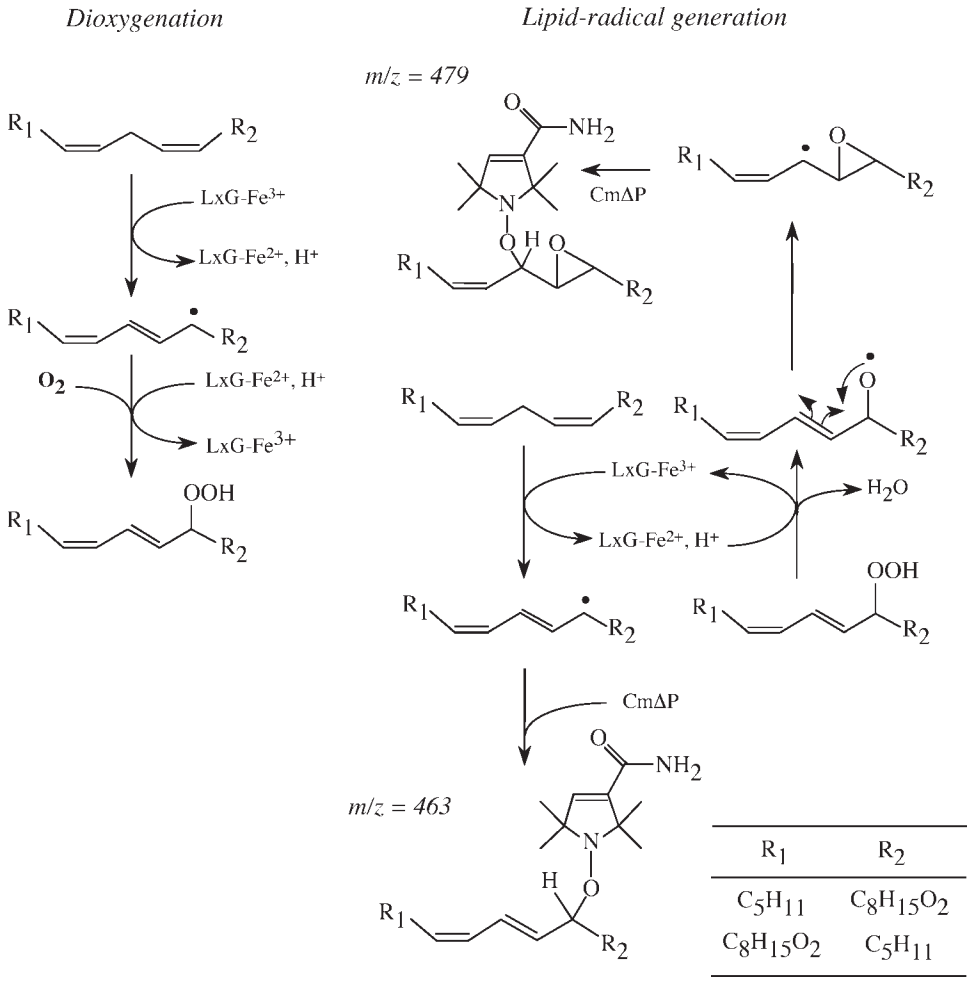
Lipid-derived radicals generated chemically from linoleic acid and linolelaidic acid were trapped with CmΔP as follows. One milliliter of acetonitrile containing 10 mM linoleic acid (or linolelaidic acid), 10 mM CmΔP, and 10 mM AMVN was transferred into a brown vial tube (inner volume, 2.0 ml) with screw cap, and the vial was left to stand at 37°C for 48 h. The reaction solution was subjected to HPLC.

HPLC analyses of lipid-derived radical-nitroxyl radical adducts

The HPLC assembly consisted of a HPLC pump (PU-2080; Jasco), a sample injector (7725; Rheodyne), a ultraviolet/visible spectrophotometer (L-2420; Hitachi), and a chromatointegrator (Chromatocorder 21; SIC). The chromatographic conditions for the quantification of lipid-derived radical-nitroxyl radical adduct were as follows: TSKgel ODS-80Ts QA column (4.6 mm inner diameter × 150 mm) with TSKguardgel ODS-80Ts guard column (3.2 mm inner diameter × 15 mm); eluent, 75% acetonitrile containing 40 mM acetate buffer (pH 3.0); flow rate, 1.0 ml/min; column temperature, 25–28°C; detection, at 210 nm.

The online thermal decomposition system consisted of a reaction coil (Teflon tube; 0.5 mm inner diameter × 10 m), a dry reaction bath (RO-2061; Jasco), and a back pressure coil (Teflon tube; 0.25 mm inner diameter × 5 m). The system was followed by electrochemical detection (ECD) (ECD-300; Eicom).

The online liquid chromatography-tandem mass spectrometry (LC/MS/MS) system consisted of the Agilent 1100 HPLC sys-



Scheme 1. Possible reaction paths in the linoleic acid/lipoxygenase/3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-*N*-oxyl (CmΔP) system. An excess amount of linoleic acid over oxygen content is present in this system.

tem and Q TRAP® LC/MS/MS system (Applied Biosystems/MDS Sciex, Concord, Ontario, Canada) equipped with an electrospray ion source. The liquid chromatograph flow rate (1.0 ml/min) into the mass spectrometer inlet was adjusted to 200 μl/min with a splitter.

Quantification of lipid-derived radical-nitroxyl radical adducts by thermal decomposition

Reaction solution containing carbon-centered alkyl radical-CmΔP adducts was diluted twice with acetonitrile. The solution was transferred into a glass vial with screw cap and heated at 100°C. The solutions were subjected to ESR spectrometry. The conditions for the ESR measurements were as follows: microwave power, 10 mW; frequency, 9.430 GHz; magnetic field, 339.5 ± 7.5 mT; modulation, 100 kHz, 0.2 mT; time constant, 0.3 s.

RESULTS AND DISCUSSION

Thermal decomposition of nitroxyl radical-lipid alkyl radical adducts

The active ferric form of lipoxygenase can catalyze the abstraction of hydrogen from the bis-allylic carbon atom

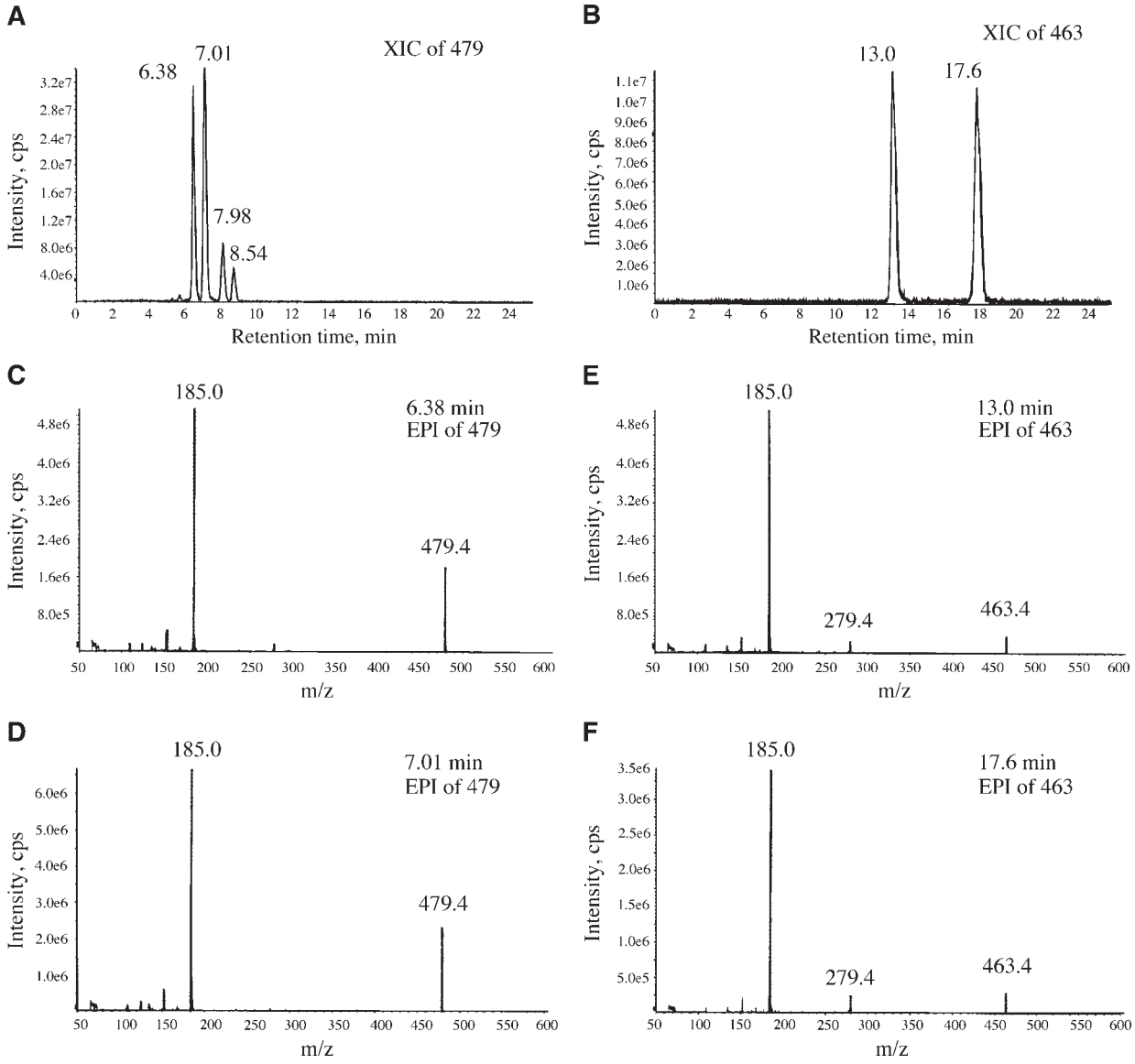


Fig. 1. Liquid chromatography-tandem mass spectrometry (LC/MS/MS) analyses of nitroxyl radical-linoleate carbon-centered radical adducts. Forty microliters of 0.1 M phosphate buffer (pH 7.4) containing 1.0 mM linoleic acid, 1.0 mM CmΔP, and 1.0 μM soybean lipoxygenase-1 was left to stand in a glass vial tube with screw cap (inner volume, 0.5 ml) at room temperature for 10 min. The reaction solution was mixed with 160 μl of acetonitrile, and then the solution was centrifuged at 10,000 *g* for 5 min. The supernatant was subjected to LC/MS/MS. A: Extracted Ion Chromatogram (XIC) of *m/z* 479 ($[M+H]^+$) from LC/MS data of CmΔP-linoleate carbon-centered radical adducts. B: XIC of *m/z* 463 from LC/MS data of CmΔP-linoleate carbon-centered radical adducts. C: MS/MS of protonated molecule of *m/z* 479 in MS of adduct 1 (6.38 min). D: MS/MS of protonated molecule of *m/z* 479 in MS of adduct 2 (7.01 min). E: MS/MS of protonated molecule of *m/z* 463 in MS of adduct 3 (13.02 min). F: MS/MS of protonated molecule of *m/z* 463 in MS of adduct 5 (17.58 min). It should be noted that the nitroxyl radical-linoleate alkyl radical adducts degrade into hydroxylamine (*m/z* 185 for CmΔP) in the collision cell of the mass spectrometer.

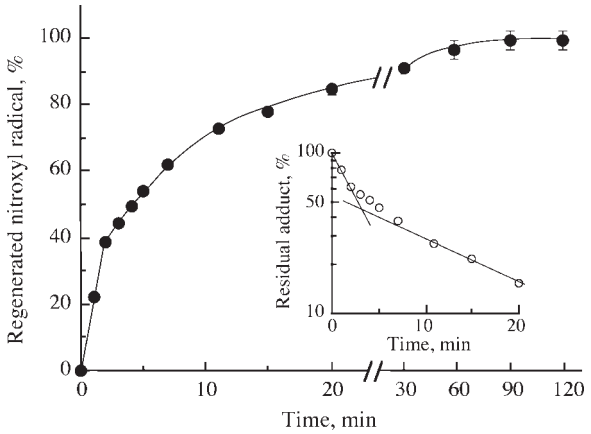


Fig. 2. Thermal decomposition of CmΔP-linoleate carbon-centered radical adducts into the corresponding nitroxyl radical. Phosphate buffer (0.1 M; pH 7.4) containing 1.0 mM linoleic acid, 200 μM CmΔP, and 1.0 μM soybean lipoxygenase-1 was left to stand in a glass vial tube with screw cap (inner volume, 0.5 ml) at room temperature for 10 min. The residual CmΔP was <1% of initial CmΔP content. The solution was diluted twice with acetonitrile, and then the solution was heated at 100°C. The solution was subjected to X-band ESR. The ESR conditions are described in Experimental Procedures. Error bars represent standard deviation.

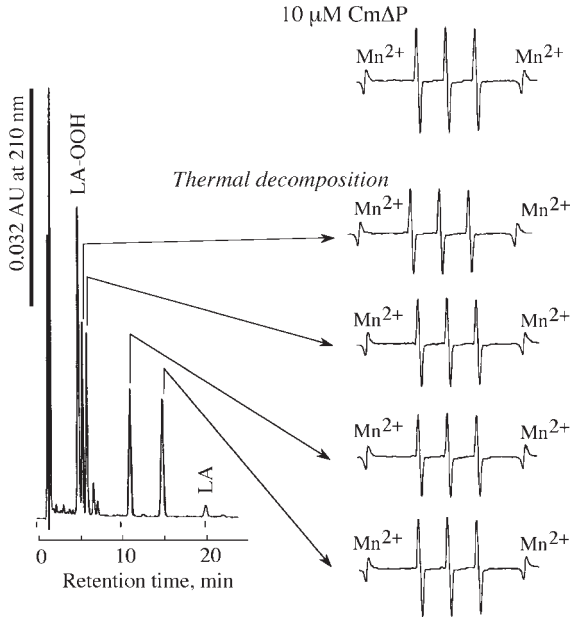
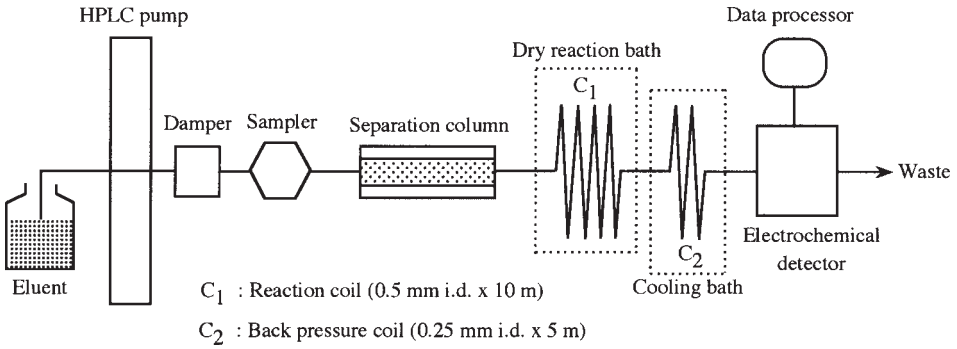


Fig. 3. Preparation and standardization of CmΔP-linoleate carbon-centered radical adducts produced in the linoleic acid/lipoxygenase/CmΔP system. Forty microliters of 0.1 M phosphate buffer (pH 7.4) containing 1.0 mM linoleic acid, 0.2 mM CmΔP, and 1.0 μM soybean lipoxygenase-1 was left to stand in a glass vial tube with screw cap (inner volume, 0.5 ml) at room temperature for 10 min. The reaction solution was mixed with 160 μl of acetonitrile, and then the solution was centrifuged at 10,000 *g* for 5 min. The supernatant (100 μl; a 20 μl aliquot was injected in each fractionation) was subjected to HPLC with ultraviolet detection. Each adduct was fractionated, and then the fractions were dried under argon gas. The residues were suspended in 50% acetonitrile. A part of the suspension was heated at 100°C for 2 h, and then the solutions were subjected to ESR spectrometry. AU, absorbance units; LA-OOH, hydroperoxy linoleic acid; LA, linoleic acid.

of the polyunsaturated fatty acid in a stereospecific manner, yielding a pentadienyl radical complexed with the ferrous enzyme (21). At lower oxygen concentrations, nitroxyl radical traps the radical intermediate on the enzyme, yielding the ferrous form of lipoxygenase. If hydroperoxy fatty acid coexists in this reaction system, the ferrous form of lipoxygenase is converted into the active ferric form, generating alkoxyl radical (22–27). This oxygen-centered alkoxyl radical is immediately converted into carbon-centered epoxyallyl radical in aqueous solution through intramolecular rearrangement (28, 29). Nitroxyl radical can trap these carbon-centered radicals, generating equimolar amounts of the nitroxyl radical-lipid alkyl radical adducts and the nitroxyl radical-lipid epoxyallyl radical adducts. The overall paths are shown in **Scheme 1**.

When 0.1 M phosphate buffer (pH 7.4; treated with Chelex 100) containing 1 mM linoleic acid, 1 μM soybean lipoxygenase-1, and 200 μM CmΔP was left to stand in a glass vial tube with screw cap at room temperature for 10 min, the residual CmΔP in the reaction system was <2 μM. The reaction solution was diluted five times with acetonitrile, and then the solution was centrifuged at 10,000 *g* for 10 min. The generation of nitroxyl radical-lipid alkyl radical (*m/z* 463) and nitroxyl radical-lipid epoxyallyl radical (*m/z* 479) in the reaction system was confirmed by LC/MS/MS analysis, as shown in **Fig. 1**.



Scheme 2. Diagram of the HPLC/electrochemical detection (ECD) system with online postcolumn thermal decomposition. i.d., inner diameter.

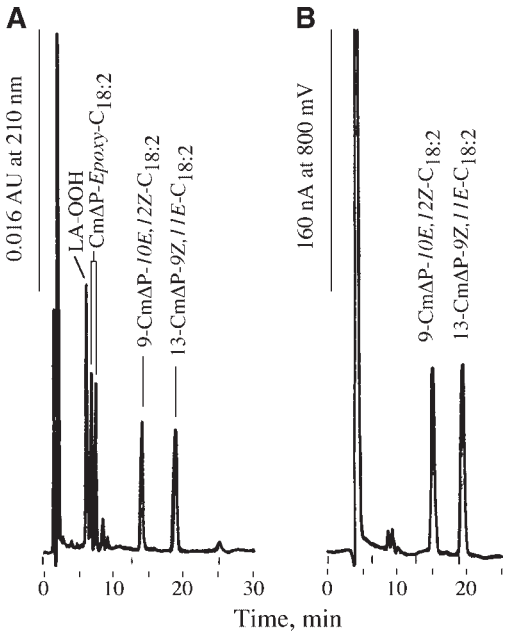


Fig. 4. Chromatographic analyses of CmΔP-linoleate carbon-centered radical adducts. A: HPLC with ultraviolet detection at 210 nm. B: HPLC/ECD with online postcolumn thermal decomposition. The HPLC conditions are described in Experimental Procedures. AU, absorbance units.

The supernatant of the reaction solution was further diluted twice with water. A portion of the solution (0.2 ml) was transferred into a glass vial with screw cap, and the vial was heated at 100°C. After a certain time interval, ESR spectra of regenerated CmΔP in the solution were measured. The time course of the relative recovery of CmΔP is shown in **Fig. 2**. It is remarkable that the thermal decomposition of CmΔP-lipid-derived radical adducts proceeded in two phases: early and late decomposition. As shown in Fig. 2 (inset), curve-fitting evaluation indicates that ~50% of CmΔP-lipid-derived radical adducts degraded rapidly, and the other 50% degraded gradually. The labile adduct was estimated to degrade by >80% within 2 min at 100°C.

As shown in Fig. 2, CmΔP-lipid-derived radical adducts were completely degraded into CmΔP by heating at 100°C for 2 h. Four CmΔP-lipid-derived radical adducts were fractionated by HPLC, and then each adduct was standardized via the thermal decomposition method using CmΔP as a standard substance (**Fig. 3**). Approximately 96% of adducts were recovered through the fractionation. The resulting standard solutions of CmΔP-lipid-derived radical adducts should be left to stand at −20°C.

Postcolumn thermal decomposition HPLC with ECD capacity for the detection of nitroxyl radical-lipid alkyl radical adducts

Based on this finding, we established HPLC with thermal decomposition, in which the regenerated nitroxyl radical was selectively and sensitively detected by ECD. A diagram of the HPLC apparatus is shown in **Scheme 2**.

For the quantitative spin-trapping, 0.1 M phosphate buffer (pH 7.4) containing 1.0 mM linoleic acid, 1 μM soybean lipoxygenase-1, and 1.0 mM CmΔP was left to stand at room temperature for 10 min, and then the solution was diluted five times with cold acetonitrile. After centrifugation at 10,000 *g* for 5 min, the supernatant was subjected to HPLC with ultraviolet detection at 210 nm. The chromatogram is shown in **Fig. 4A**. Approximately equal amounts of four adducts were detected: 9-CmΔP-10*E*,12*Z*-$C_{18:2}$, 13-CmΔP-9*Z*,11*E*-$C_{18:2}$, and two regioisomers of nitroxyl radical-linoleate epoxyallyl radical adducts (CmΔP-epoxy-$C_{18:2}$). Similarly, the supernatant was subjected to HPLC-ECD with postcolumn thermal decomposition. The chromatogram is shown in Fig. 4B. Remarkably, the nitroxyl radical-lipid alkyl radical adducts 9-CmΔP-10*E*,12*Z*-$C_{18:2}$ and 13-CmΔP-9*Z*,11*E*-$C_{18:2}$ were detected. However, in this system,

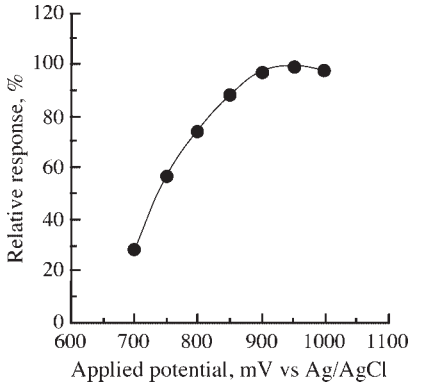


Fig. 5. Effect of applied potential on the electrochemical detection of the CmΔP-linoleate alkyl radical adducts. The reaction solution of the linoleic acid/lipoxygenase/CmΔP system was subjected to HPLC/ECD with online postcolumn thermal decomposition. The relative response (%) represents the relative peak area against that detected at 950 mV versus Ag/AgCl.

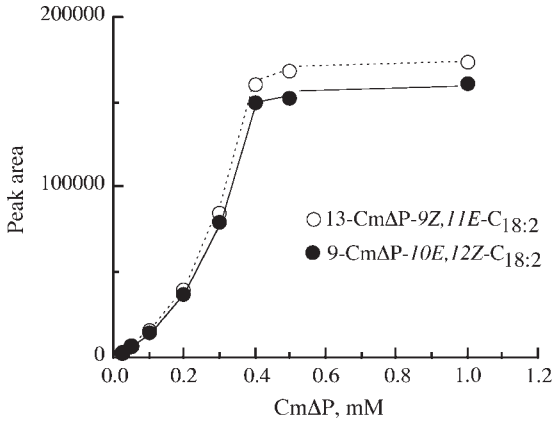


Fig. 6. Effect of CmΔP content on the spin-trapping of linoleate alkyl radical. Forty microliters of 0.1 M phosphate buffer (pH 7.4) containing 1.0 mM linoleic acid, CmΔP, and 1.0 μM soybean lipoxygenase-1 was left to stand in a glass vial tube with screw cap (inner volume, 0.5 ml) at room temperature for 10 min. The reaction solution was mixed with 160 μl of acetonitrile, and then the solution was centrifuged at 10,000 *g* for 5 min. The supernatant was subjected to HPLC/ECD with online postcolumn thermal decomposition (800 mV vs. Ag/AgCl).

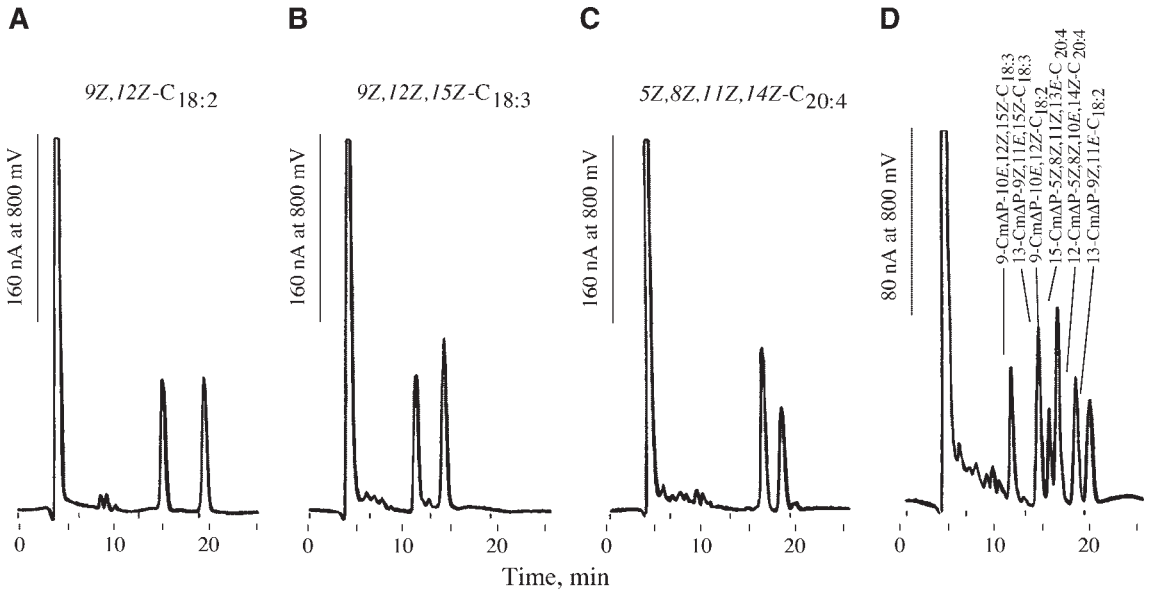


Fig. 7. Chromatographic evaluation of lipid alkyl radical generation from linoleic acid (9Z,12Z-$C_{18:2}$), linolenic acid (9Z,12Z,15Z-$C_{18:3}$), arachidonic acid (5Z,8Z,11Z,14Z-$C_{20:4}$), or a mixed sample via soybean lipoxygenase-1. One millimolar linoleic acid (A), 1.0 mM linolenic acid (B), 1.0 mM arachidonic acid (C), and a mixed sample (0.33 mM each; D) were treated with 1.0 μM soybean lipoxygenase-1 in the presence of 1.0 mM CmΔP. The HPLC conditions are described in Experimental Procedures. Detection was at 800 mV versus Ag/AgCl. Sample volume was 10 μl (A–C) and 15 μl (D).

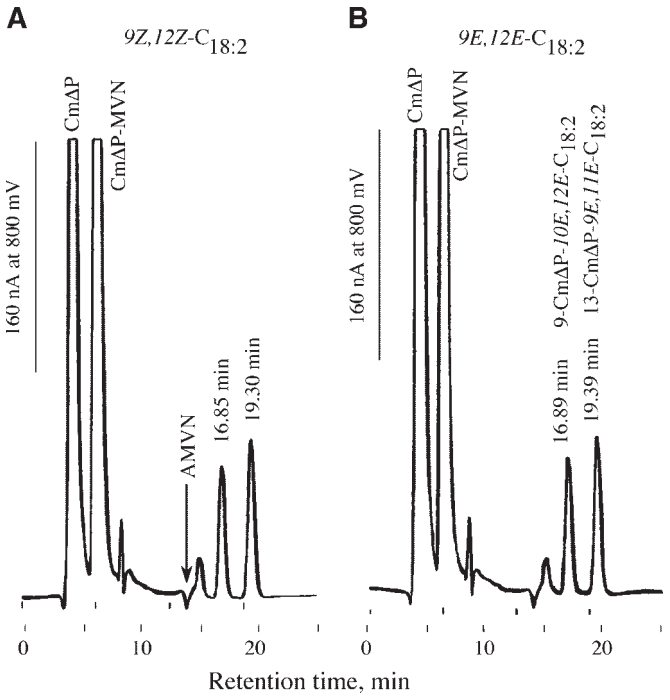


Fig. 8. Chromatographic analyses of CmΔP-lipid alkyl radical adducts generated from linoleic acid (9Z,12Z-$C_{18:2}$) and linolelaidic acid (9E,12E-$C_{18:2}$) via the radical initiator 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN). Acetonitrile solution containing 10 mM CmΔP, 10 mM AMVN, and 10 mM linoleic acid (A) or linolelaidic acid (B) was left to stand at 37°C for 48 h. One volume of the reaction solution was mixed with 19 volumes of acetonitrile, and a 10 μl portion of the solution was subjected to HPLC/ECD with online postcolumn thermal decomposition (800 mV vs. Ag/AgCl).

CmΔP-epoxy-$C_{18:2}$ content was extremely low. Through these findings, it appeared that nitroxyl radical-lipid epoxyallyl radical adducts are comparatively resistant to thermal decomposition. **Figure 5** shows the correlation between the applied potential and the relative response. To quantitatively trap lipid alkyl radicals, we estimated an optimal nitroxyl radical content. As shown in **Fig. 6**, CmΔP content >0.5 mM was required for the quantification of linoleate alkyl radicals generated in the linoleic acid/lipoxygenase/CmΔP system.

Quantitative evaluation of chemically and enzymatically generated lipid alkyl radicals from polyunsaturated fatty acid

Linoleic acid (9Z,12Z-$C_{18:2}$), linolenic acid (9Z,12Z,15Z-$C_{18:3}$), and arachidonic acid (5Z,8Z,11Z,14Z-$C_{20:4}$) are the principal polyunsaturated fatty acids found in organisms. Lipoxygenases are believed to convert fatty acids into specific oxidation products. In the present study, we evaluated the lipid alkyl radical generation from these individual polyunsaturated fatty acids and their mixture via soybean lipoxygenase-1. The chromatograms are shown in **Fig. 7**. In common, the generation of two regioisomers of CmΔP-lipid alkyl radical adducts from each individual fatty acid was confirmed. Nitroxyl radical competes with the oxygen molecule for lipid alkyl radical on soybean lipoxygenase-1. Consequently, CmΔP-lipid alkyl radical adducts should resemble hydroperoxy fatty acids generated at lower oxygen content in regiospecificity and stereospecificity. Regarding the regiospecificity of hydroperoxy fatty acids generated in polyunsaturated fatty acid/soybean lipoxygenase-1

systems (30–32), the regiospecificity and stereospecificity of CmΔP-lipid alkyl radical adducts were identified as follows: 13-CmΔP-9*Z*,11*E*-$C_{18:2}$ (major) and 9-CmΔP-10*E*,12*Z*-$C_{18:2}$ (minor) from linoleic acid; 13-CmΔP-9*Z*,11*E*,15*Z*-$C_{18:3}$ (major) and 9-CmΔP-10*E*,12*Z*,15*Z*-$C_{18:3}$ (minor) from linolenic acid; 15-CmΔP-5*Z*,8*Z*,11*Z*,13*E*-$C_{20:4}$ (major) and 12-CmΔP-5*Z*,8*Z*,10*E*,14*Z*-$C_{20:4}$ (minor) from arachidonic acid. The satisfactory separation of these adducts was achieved by reversed-phase HPLC, as shown in Fig. 7D.

In a similar manner, we tried to trap lipid alkyl radicals, which were chemically generated from linoleic acid or linolelaidic acid via the lipophilic radical initiator AMVN. One milliliter of acetonitrile containing 10 mM linoleic acid or linolelaidic acid, 10 mM CmΔP, and 10 mM AMVN [half-life at 37°C, 90 h (33)] was left to stand in a glass vial with screw cap (inner volume, 2 ml) at 37°C for 48 h. The reaction solutions were diluted 20 times with acetonitrile, and the solutions were subjected to HPLC. The chromatograms are shown in **Fig. 8**. Two main adducts (*m/z* 463) were detected in both systems. The intermediate carbon-centered *E/Z*-pentadiene radical undergoes resonance stabilization into two positionally isomeric pentadiene radicals, C-9 and C-13 positions. In a nonenzymatic reaction, the *cis* double bond of the radicals can spontaneously isomerize to a thermodynamically favored *trans* configuration (34). Consequently, despite the difference in the stereospecificity of precursor fatty acids, the stereospecificities of the resulting CmΔP-lipid alkyl radical adducts were identical.

We succeeded, for the first time, in establishing an HPLC system for the sensitive and selective quantification of lipid alkyl radicals generated through chemical and enzymatic hydrogen abstraction. The HPLC system can be easily assembled from commercially available equipment in scientific laboratories. We expect that this method will be applied to the assessment of lipid alkyl radical generation in biological systems.

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