Intramolecular rearrangement of linolenate peroxyl radicals in lipoxygenase reactions at lower oxygen content

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Abstract **Variation of tissue oxygen content is thought to be a possible factor in determining the structural diversity of hydroperoxy fatty acids. In the present study, we evaluated the structural diversity of intermediate carbon-centered radicals at lower oxygen content. When the buffered solution (pH 7.4) containing 1.0 mM α-linolenic acid, 1.0 μM soybean 15-lipoxygenase, and 1.0 mM nitroxyl radical [3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-*N*-oxyl (CmΔP)], which selectively traps carbon-centered radicals, was incubated in a sealed vial, the generation of linolenate hydroperoxide was completed within 1 min. In the subsequent reaction at lower oxygen content, the production of the [LnA−H+O_2]·-CmΔP adduct was ascertained by liquid chromatography tandem mass spectrometry with precursor ion scanning. Furthermore, HPLC analysis with photodiode array detection showed that the adduct exhibits an absorption maximum at 278 nm, indicating a conjugated triene moiety. On the basis of these facts, the structure of the adduct was speculated to be C_2H_5−CH(CmΔP)−CH = CH−CH = CH−CH = CH−CH(OOH) −C_7H_{14}−COOH. We proposed a possible reaction pathway as follows: a linolenate 9-peroxyl radical generated in the lipoxygenase reaction might be converted into C_2H_5−·CH−CH = CH−CH = CH−CH = CH−CH(OOH) −C_7H_{14}−COOH through an intramolecular rearrangement. This intermediate radical may give rise to hydroperoxy fatty acids with structural diversity.**—Takajo, T., K. Tsuchida, T. Murahashi, K. Ueno, and I. Koshiishi. **Intramolecular rearrangement of linolenate peroxyl radicals in lipoxygenase reactions at lower oxygen content. *J. Lipid Res.* 2007.** 48: **855–862.**

Supplementary key words carbon-centered radical • spin-trapping • mass spectrometry • precursor ion scanning

It is generally accepted that lipoxygenase, in combination with phospholipase A_2, is involved in ischemia-reperfusion injury (1–6). However, the real mechanism of how the tissue is injured via lipoxygenase and polyunsaturated fatty acids (PUFAs) remains obscure. Furthermore, there are few examples of structural diversity in the resulting hydroperoxy fatty acids and the reaction pathway under ischemic conditions.

Lipoxygenases insert an oxygen molecule into an individual position in PUFAs including arachidonic acid, eicosapentaenoic acid, linoleic acid, and α-linolenic acid. 15-Lipoxygenase and 5-lipoxygenase are the principal isozymes involved in inflammatory responses. At the initial stage of lipid peroxidation by lipoxygenase, a doubly allylic hydrogen should be abstracted from PUFA, which is accompanied by reduction of ferric lipoxygenase to the ferrous form. It seems that an oxygen molecule can reach the carbon-centered radicals through a channel in the lipoxygenase, resulting in the highly controlled stereo- and regiospecificities of the resultant hydroperoxy fatty acid (7). In general, decreased oxygen content results in a lack of regiospecificity (8). In fact, it was demonstrated that 15-lipoxygenase inserts an oxygen molecule into linoleic acid and α-linolenic acid at the C-9 position as well as the C-13 position at lower oxygen content (9–11). This fact indicates that the binding affinity of lipid allyl radicals to the lipoxygenase should be loose at lower oxygen content as compared with that in normoxia.

In order to evaluate the structural diversity of hydroperoxy fatty acids produced in the PUFA/lipoxygenase system at lower oxygen content, it is effective to evaluate the generation of the fatty acid-derived carbon-centered radicals with which the oxygen molecule can react. Furthermore, the evaluation of the intermediate carbon-centered radical generation could lead to the elucidation of the reaction pathway. For this purpose, we established a novel method for the detection of fatty acid-derived

Abbreviations: CmΔP, 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-*N*-oxyl; LC, liquid chromatography; MS/MS, tandem mass spectrometry; PIS, precursor ion scanning; UV, ultraviolet.

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carbon-centered radicals via the combination of the nitroxyl radical spin-trapping technique with the precursor ion-scanning (PIS) technique in liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. The nitroxyl radical, 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-*N*-oxyl (CmΔP), was employed to selectively trap carbon-centered radicals, although the resulting adducts are comparatively thermolabile (12).

In the present study, we set up an in vitro hypoxia condition with soybean 15-lipoxygenase and α-linolenic acid: Oxygen was consumed by the dioxygenation of PUFA in the lipoxygenase reaction. The fatty acid-derived carbon-centered radical-CmΔP adducts produced in the linolenate/lipoxygenase system in the presence of CmΔP were identified by LC-MS/MS with PIS and HPLC with photodiode array detection. And then, on the basis of the adduct structure, we speculated a possible pathway for fatty acid-derived carbon-centered radical generation at lower oxygen content.

EXPERIMENTAL PROCEDURES

Materials

Soybean lipoxygenase-1 (type I-b; activity, 70,800 U/mg; molecular mass, 108 kDa), linoleic acid, and α-linolenic acid were purchased from Sigma. 13-Hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). CmΔP was purchased from Aldrich Chemical Co., Inc. and was 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. All other chemicals used were reagent grade.

If necessary, α-linolenic acid was chromatographically purified as follows: 2 ml of 50 mM α-linolenic acid in 50% acetonitrile was passed through two Sep-Pak Plus C18 columns (Waters Co.). α-Linolenic acid on the columns was eluted by water-acetonitrile gradient elution. These operations were performed in a nitrogen atmosphere. Contaminant linolenate hydroperoxide was eluted in front of α-linolenic acid. The α-linolenic acid fraction was evaporated, and the residue was dissolved in ethanol. The concentration was adjusted to 100 mM, and the solution was stored at −80°C.

Lipid-derived radical trapping by nitroxyl radical

Lipid-derived radicals generated in the PUFA/lipoxygenase system were trapped with the nitroxyl radical CmΔP as follows: 20 μl of 2 mM PUFA 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,

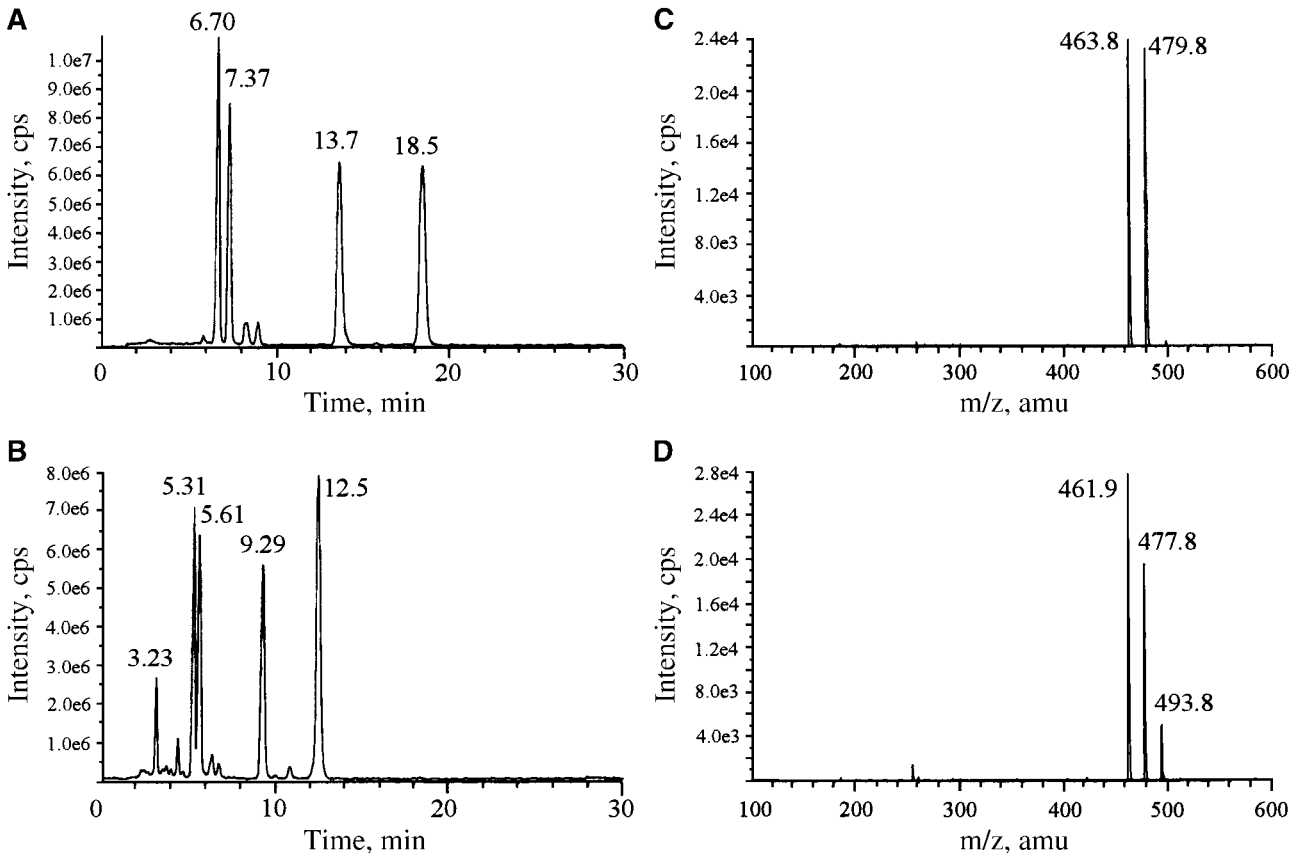

Fig. 1. Liquid chromatography tandem mass spectrometry (LC-MS/MS) with precursor ion scanning (PIS) for 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-*N*-oxyl (CmΔP) spin-trapping adducts in PUFA/lipoxygenase/CmΔP systems. A, B: The total ion chromatograms of adducts detected by PIS for *m/z* 185. C, D: The cumulative mass spectra of precursor ions corresponding to $[M+H]^+$ of lipid-derived radical-CmΔP adducts eluted from 0 min to 30 min. A, C: linoleate/lipoxygenase/CmΔP system; B, D: linolenate/lipoxygenase/CmΔP system.

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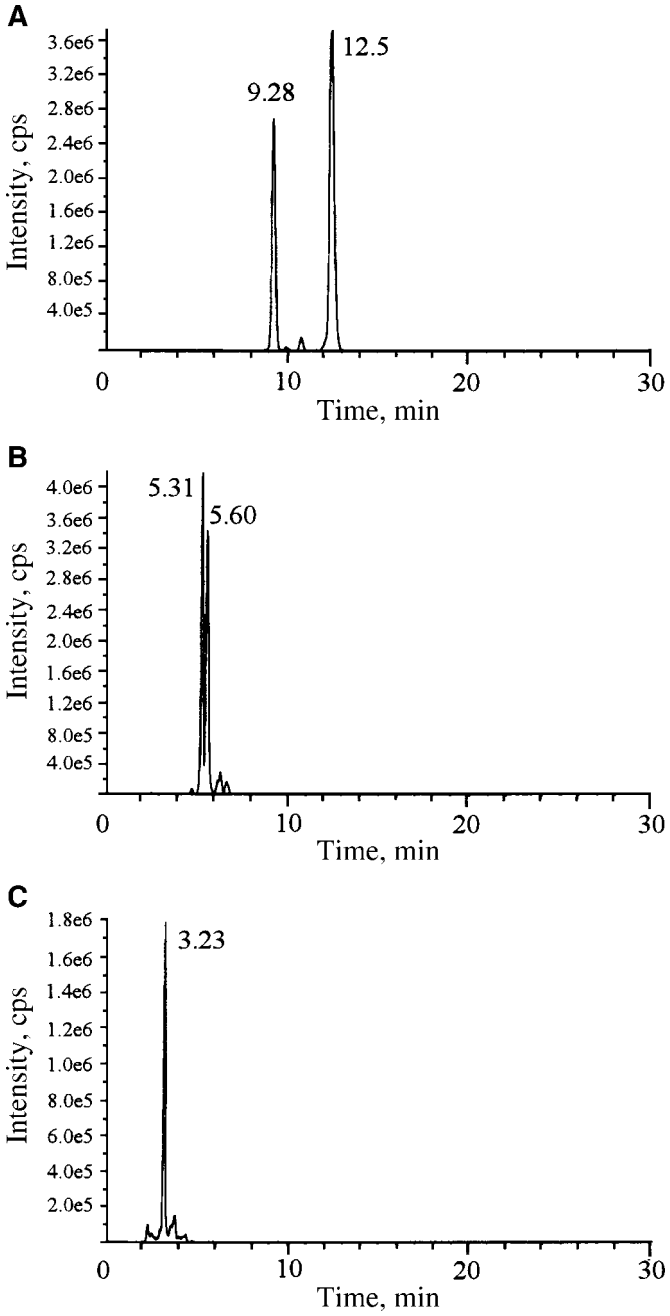

Fig. 2. Extracted ion chromatograms in LC-MS/MS with PIS for *m/z* 185. A: CmΔP spin-trapping adducts of carbon-centered radicals corresponding to linolenate allyl radicals. B: CmΔP spin-trapping adducts of carbon-centered radicals corresponding to linolenate epoxyallyl radicals. C: CmΔP spin-trapping adducts of carbon-centered radicals corresponding to $[LnA-H+O_2]\cdot$.

0.5 ml), which was incubated at 25°C for 5 min. The reaction solution was mixed with 160 μl of cold acetonitrile and then centrifuged at 10,000 *g* for 1 min at 0°C. The supernatant was subjected to HPLC.

HPLC analyses of lipid-derived radical-nitroxyl radical adducts

The chromatographic conditions for the quantification of the lipid-derived radical-nitroxyl radical adducts are as follows: column, TSKgel ODS-80Ts QA (4.6 mm i.d. × 150 mm) with guard column, TSKguardgel ODS-80Ts (3.2 mm i.d. × 15 mm); eluent, 75% acetonitrile containing 0.05% formic acid; flow rate, 1.0 ml/min; column temperature, 25–28°C. The on-line LC-MS/MS system consisted of the Agilent1100 HPLC system and API 4000® Triple Quadrupole LC-MS/MS system (Applied Biosystems/MDS Sciex; Concord, ON, Canada) equipped with an electrospray ion source. Tandem mass spectrometry (MS/MS) conditions for API-4000 are as follows: polarity, positive; curtain gas, 50 psi; ion source gas 1, 30 psi; ion source gas 2, 70 psi; ion spray voltage, 5,500 V; temperature, 600°C; collision gas, 1.00; declustering potential, 81 V; entrance potential, 10 V; collision cell exit potential, 15 V; collision energy, 30 V; channel electron multiplier, 2,000 V; deflector, −100 V. The HPLC with photodiode array detection system consisted of a pump (LC-20AD; Shimadzu, Japan), a degasser (DGU-20A5; Shimadzu), a photodiode array detector (GL-7452; GL Science, Japan), a column oven (CTO-20A; Shimadzu), and an auto-sampler (SIL-20A, Shimadzu).

HPLC analyses of PUFAs and their hydroperoxides

The chromatographic conditions for the quantification of the PUFAs and hydroperoxides were the same as those for the quantification of the lipid-derived radical-nitroxyl radical adducts. PUFAs and hydroperoxides were detected at 210 nm and 234 nm, respectively.

Effects of coexisting hydroperoxides on the generation of the lipid-derived radical-nitroxyl radical adducts

Lipid-derived radicals generated in the linolenate/lipoxygenase system in the presence of 13-hydroperoxy-octadecadienoic acid were trapped with the nitroxyl radical CmΔP as follows: 10 μl of 4 mM α-linolenic acid emulsion in 0.1 M phosphate buffer (pH 7.4, treated with Chelex 100) containing 4% ethanol was mixed with 10 μl of 13-hydroperoxy-octadecadienoic acid in 0.1 M phosphate buffer (pH 7.4, treated with Chelex 100), 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), which was incubated at 25°C for 5 min. The reaction solution was mixed with 160 μl of cold acetonitrile and then centrifuged at 10,000 *g* for 1 min at 0°C. The supernatant was subjected to HPLC with ultraviolet (UV) detection.

RESULTS AND DISCUSSION

In the present study, soybean lipoxygenase-1 (type I-b) and α-linolenic acid were employed as 15-lipoxygenase and PUFA, respectively, to evaluate the generation of fatty acid-derived carbon-centered radicals in a lipoxygenase reaction at lower oxygen content. To establish the lower oxygen condition, we employed a linolenate/lipoxygenase system, in which dissolved oxygen (approximately 230 μM) is consumed by the dioxygenation of α-linolenic acid. When the phosphate buffer solution (0.1 M, pH 7.4) containing 1.0 mM α-linolenic acid and 1.0 μM lipoxygenase was incubated in a sealed vial at 25°C, the linolenate hydroperoxide content reached a plateau within 1 min (data not shown).

To detect lipid-derived carbon-centered radicals, it is essential for the spin-trapping agent to trap every type of carbon-centered radical. In our recent study (12), we demonstrated that the nitroxyl radical, CmΔP: M.W., 183, stoichiometrically trapped lipid-derived carbon-centered

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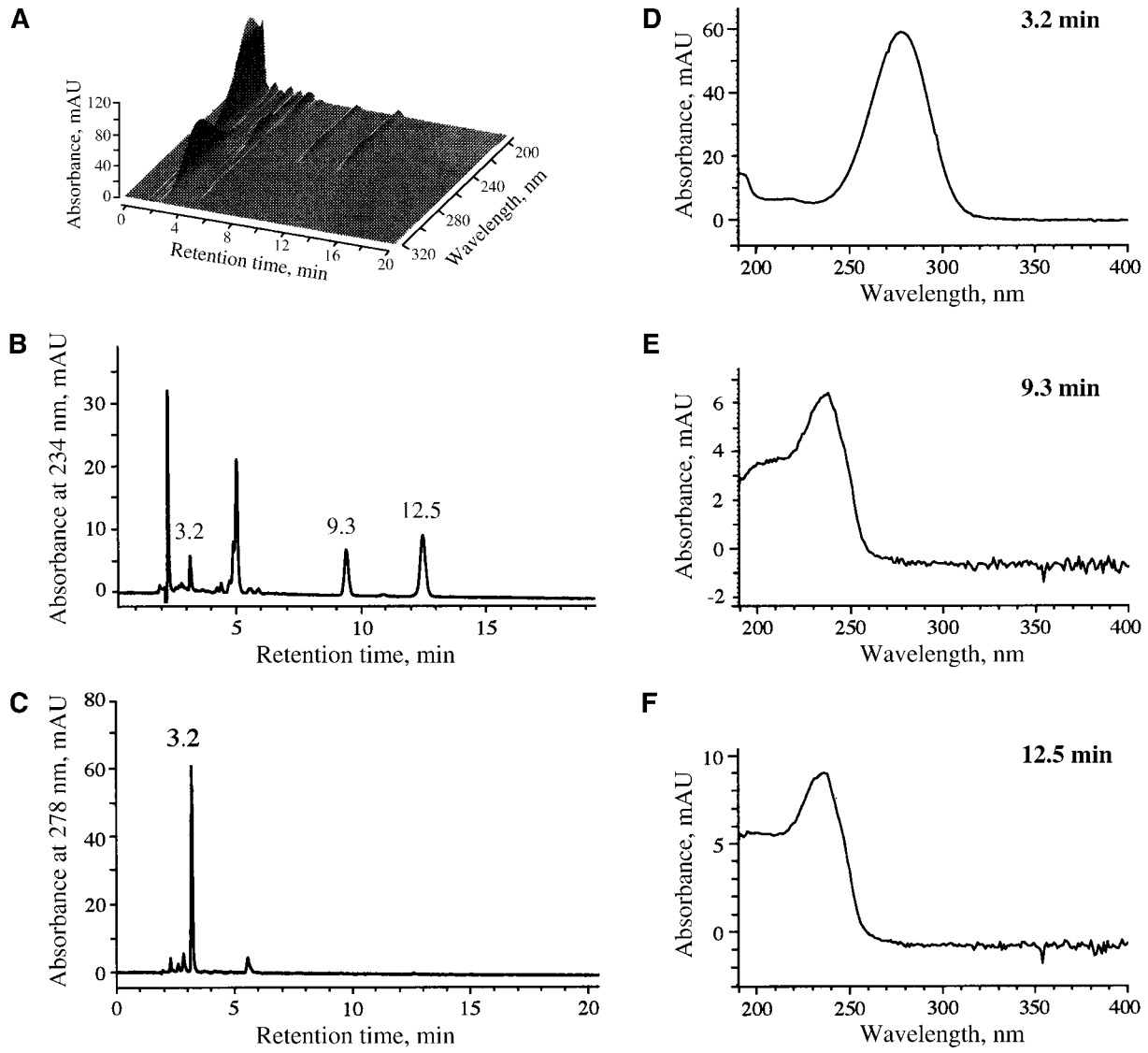

Fig. 3. Ultraviolet (UV) spectra of CmΔP spin-trapping adducts of carbon-centered radicals produced in the linolenate/lipoxygenase/CmΔP system. Reaction solution was subjected to HPLC with photodiode array detection. A: 3-D elution profile. B, C: Chromatograms of CmΔP spin-trapping adducts detected at 234 nm and 278 nm, respectively. D, E, F: UV spectra of CmΔP spin-trapping adducts eluted at 3.2 min, 9.3 min, and 12.5 min, respectively.

radicals, including lipid allyl radicals and lipid epoxyallyl radicals, in the linoleate/lipoxygenase system through radical-radical conjunction. We confirmed that the presence of the nitroxyl radical did not prevent the lipoxygenase reaction as follows: when 1.0 mM linoleate was incubated with 0.1 μM lipoxygenase in the presence or absence of CmΔP (1.0 mM), there is no significant difference in the initial rate of hydroperoxide generation between the two systems (approximately 2.4 μM/sec). Remarkably, the nitroxyl radical scavenges the linoleate allyl radical on the ferrous lipoxygenase at lower oxygen content, resulting in the release of the inactive ferrous lipoxygenase (13). It is generally known that lipid hydroperoxides play a key role in the conversion of ferrous lipoxygenase into active ferric lipoxygenase, generating lipid alkoxyl radicals (14–16), which easily turn into epoxyallyl radicals through intramolecular rearrangement.

Interestingly, MS/MS spectra of these adducts showed that carbon-centered radical-CmΔP adducts characteristically fragmented into hydroxylamine (*m/z* 185), which arises from the reduced form of CmΔP through heterolysis of the C-O-N bond (12). This characteristic fragmentation of lipid-derived carbon-centered radical-CmΔP adducts should lead to the selective detection of adducts via MS/MS with PIS (MS/MS-PIS) for *m/z* 185. With this phenomenon in mind, we tried to detect nitroxyl radical spin-trapping adducts of lipid-derived carbon-centered radicals generated from linoleic acid and α-linolenic acid through the lipoxygenase reaction by using liquid chromatography mass spectrometry/mass spectrometry precursor ion scanning (LC-MS/MS-PIS). The total ion chromatograms of adducts detected by the PIS for *m/z* 185 are shown in **Fig. 1A, B**. The cumulative mass spectra of the precursor ions that correspond to lipid-derived carbon-centered

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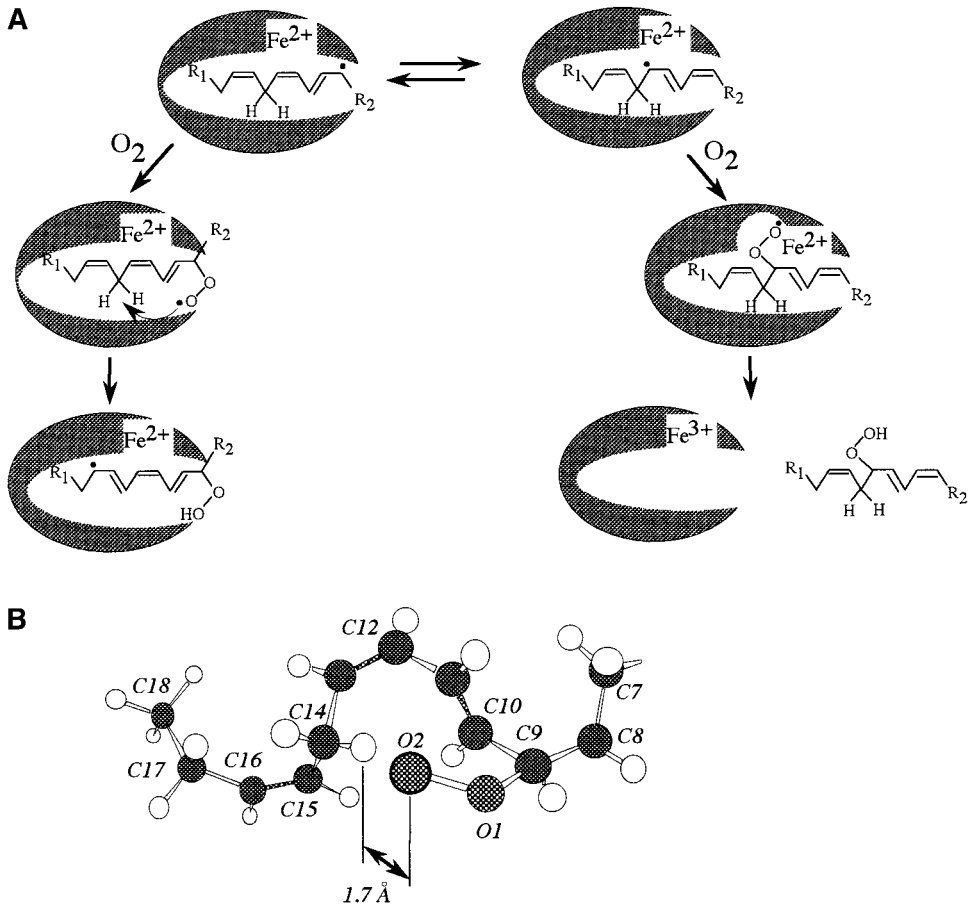

Fig. 4. A: Possible reaction pathway for the generation of the linolenate hydroperoxy allyl radical. B: 3-D structure of the linolenate 9-peroxyl radical. The unpaired electron is located on the oxygen atom at O-2.

radical-CmΔP adducts eluted from 0 min to 30 min are shown in Fig. 1C, D. In both cases, lipid allyl radical-CmΔP adducts and lipid epoxyallyl radical-CmΔP adducts were detected. The scavenging of lipid allyl radicals on the ferrous lipoxygenase by the nitroxyl radical promotes the one-electron redox cycle reaction (13).

The [LnA−H+O_2]·-CmΔP adduct was detected in the linolenate/lipoxygenase/CmΔP system (Fig. 1D), whereas the [LA−H+O_2]·-CmΔP adduct was not detected (Fig. 1C). It should be noted that the signal intensity does not necessarily correspond to the proportion of each adduct to the total amount of adducts. The extracted ion chromatograms of linolenate-derived carbon-centered radical-CmΔP adducts are shown in **Fig. 2**. Remarkably, a principal adduct corresponding to the peak at 3.2 min was detected in the linolenate/lipoxygenase/CmΔP system. To analyze the conjugated polyene moiety in the adducts, UV spectra of the [LnA−H+O_2]·-CmΔP adduct (3.2 min) and the linolenate allyl radical-CmΔP adducts (9.3 and 12.5 min) were measured by photodiode array detection (**Fig. 3**). As expected, both linolenate allyl radical-CmΔP adducts possess a λ_{max} at 234 nm, indicating that the linolenate allyl radical-CmΔP adducts have a conjugated diene moiety (−CH = CH−CH = CH−) in their structure. In contrast, the [LnA−H+O_2]·-CmΔP adduct possesses a λ_{max} at 278 nm, indicating that the [LnA−H+O_2]·-CmΔP adduct has a conjugated triene moiety (−CH = CH−CH = CH−CH = CH−) in its structure. Taken together, *i*) a carbon-centered radical corresponding to [LA−H+O_2]· was not produced in the linoleate/lipoxygenase system; *ii*) a single carbon-centered radical corresponding to [LnA−H+O_2]· was produced in the linolenate/lipoxygenase system; and *iii*) the production of a carbon-centered radical corresponding to [LnA−H+O_2]· was accompanied by the formation of a conjugated triene moiety. It should be noted here that, unfortunately, we could not subject the isolated [LnA−H+O_2]·-CmΔP adduct to structural analyses, because the adduct is so thermolabile that it is difficult to isolate (12, 17).

Lipoxygenase abstracts a doubly allylic hydrogen from C-11 of α-linolenic acid. The intermediate carbon-centered pentadiene radical undergoes resonance stabilization into two positionally isomeric pentadiene radicals at the C-9 and C-13 positions. In normoxia, an oxygen molecule selectively attacks the C-13 position, resulting in the generation of a linolenate 13-peroxyl radical. In contrast, an oxygen molecule attacks the C-9 position as well as the C-13 position at lower oxygen content. Interestingly, CmΔP competes with the oxygen molecule for the linolenate allyl radical on the lipoxygenase at lower oxygen content. As shown in

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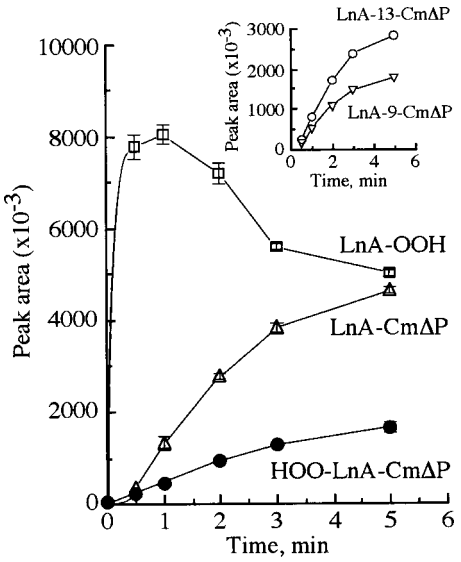

Fig. 5. Time course of linolenate hydroperoxide allyl radical-CmΔP adduct generation as compared with linolenate allyl radical-CmΔP adduct generation. At each time interval, the reaction solution was mixed with cold acetonitrile to stop the reaction, and then the solution was centrifuged at 10,000 *g* for 1 min at 0°C. An aliquot of the supernatant was injected into the HPLC. The linolenate hydroperoxide (LnA-OOH) and linolenate allyl radical-CmΔP adducts (LnA-CmΔP) were detected at 234 nm, and the linolenate hydroperoxide allyl radical-CmΔP adduct (HOO-LnA-CmΔP) was detected at 278 nm. *Inset*: Time courses of linolenate allyl radical-9-CmΔP adduct (LnA-9-CmΔP) and linolenate allyl radical-13-CmΔP adduct (LnA-13-CmΔP) eluted at 9.3 and 12.5 min, respectively. Results are mean ± SD for triplicate individual experiments.

Fig. 2A, two species of adducts corresponding to the linolenate allyl radical-9-CmΔP adduct and the 13-CmΔP regioisomer were detected by LC-MS/MS-PIS. This fact indicates that linolenate 9-peroxyl radical was generated in this linolenate/lipoxygenase system.

If α-linolenic acid is converted into a linolenate derivative possessing a conjugated triene moiety, two hydrogen atoms have to be abstracted from both of the doubly allylic methylenes at C-11 and C-14 in series. To explain the reaction of CmΔP with the carbon-centered radical corresponding to $[LnA-H+O_2]\cdot$, we speculated a mechanism as shown in **Fig. 4**. This reaction mechanism is based on the intramolecular rearrangement of the linolenate 9-peroxyl radical. In normoxia, the initially generated principal peroxyl radical is the linolenate 13-peroxyl radical, which has no doubly allylic methylene, so the peroxyl radical on the lipoxygenase must be reduced by the ferrous ion. In contrast, the peroxyl radical in the linolenate 9-peroxyl radical can abstract a hydrogen from the doubly allylic methylene at C-14, resulting in the formation of a carbon-centered radical with a conjugated triene moiety. CmΔP can react with the carbon-centered radical, resulting in the formation of $C_2H_5-\cdot CH(CmΔP)-CH=CH-CH=CH-CH=CH-CH(OOH)-C_7H_{14}-COOH$. (We abbreviate this adduct as HOO−LnA−CmΔP.)

In the present study, we propose an intramolecular rearrangement of the linolenate 9-peroxyl radical on lipoxygenase, generating the carbon-centered radical. On the other hand, if lipoxygenase can abstract the hydrogen of the doubly allylic methylene at C-14 of linolenate 9-hydroperoxide, the same intermediate carbon-centered radical, $C_2H_5-CH-CH=CH-CH=CH-CH=CH-CH(OOH)-C_7H_{14}-COOH$, should be generated. However, the possibility of this pathway should be negligible, because *i*) 15-lipoxygenase did not abstract the hydrogen of the doubly allylic methylene at C-14 of α-linolenic acid, and *ii*) excess amount of the substrate, α-linolenic acid, competes with a small amount of linolenate 9-hydroperoxide for the active site on lipoxygenase.

For an intramolecular rearrangement of the linolenate 9-peroxyl radical, the unpaired electron has to come close to the doubly allylic methylene at C-14. A three-dimensional construction of the molecule shows that the shortest distance between the doubly allylic hydrogen and the oxygen atom on which the unpaired electron is located was esti-

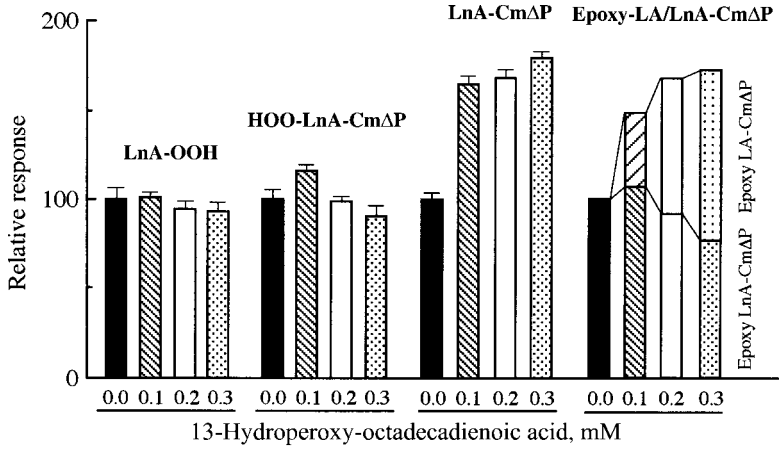

Fig. 6. Effect of the presence of 13-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid on the linolenate hydroperoxide allyl radical-CmΔP adduct generation in the linolenate/lipoxygenase/CmΔP system. Each value indicates content relative to the control value (at 0.0 mM HOO-LnA-CmΔP). Results are mean ± SD for triplicate individual experiments.

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mated to be 1.7 Å (Fig. 4B). This fact indicates that the mobility of the framework of the linolenate 9-peroxyl radical is not necessarily retarded by fixation in the active site of lipoxygenase. In our recent study, we found that the binding affinity of the lipid allyl radical to the active site on lipoxygenase at lower oxygen content is not so tight that the bulky nitroxyl radical can scavenge the lipid allyl radical (13). In a similar manner, the linolenate 9-peroxyl radical is thought to be flexible in the active site on lipoxygenase at lower oxygen content, allowing the intramolecular rearrangement of the linolenate 9-peroxyl radical.

The time course of HOO-LnA-CmΔP generation in the reaction solution is shown in **Fig. 5**. The generation of linolenate hydroperoxide was almost completed within 1 min. The dioxygenation of a PUFA results in the decreased oxygen content. In our recent study (13), we revealed that the reaction between the linoleate allyl radical and the nitroxyl radical was not observed while the dioxygenation was ongoing. As shown in Fig. 5, HOO-LnA-CmΔP was generated at lower oxygen content. Interestingly, the HOO-LnA-CmΔP content increased linearly within the initial 2 min, passing through the origin. This fact indicates that linolenate 9-peroxyl radical is generated in normoxia as well as at lower oxygen content. Even though the specific insertion of an oxygen molecule at the C-13 position of the linolenate allyl radical in normoxia is speculated to be regulated by a channel through which the oxygen molecule reaches an unpaired electron on the C-13 carbon (7), the oxygen molecule seems to partially react with the carbon-centered radical at the C-9 position of the linolenate allyl radical. Furthermore, this regiospecific regulation system in normoxia is thought to be nonfunctional at lower oxygen content. In fact, the ratio between 13-CmΔP-linolenate (LnA-13-CmΔP) and 9-CmΔP-linolenate (LnA-9-CmΔP) was almost constant at 60:40 (mol/mol).

In our hypothesis, the intramolecular rearrangement of the linolenate 9-peroxyl radical may occur on the lipoxygenase. So we have to exclude the possibility that fatty acid-derived radicals released during the catalytic activity of lipoxygenase participate in the formation of a conjugated triene moiety through a reaction between a radical and a hydroperoxide. Furthermore, Garssen, Vliegenthart, and Boldingh (18, 19) demonstrated that in an anaerobic reaction among lipoxygenase, linoleic acid, and its hydroperoxides, the linoleate allyl radical on the lipoxygenase abstracts a hydrogen from linoleate hydroperoxide, generating the linoleate peroxyl radical. To exclude the possibility of linolenate peroxyl radical generation from free linolenate hydroperoxide, we examined whether the presence of linoleate hydroperoxide [13-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid] influences the generation of HOO-LnA-CmΔP. As shown in **Fig. 6**, the linoleate hydroperoxide did not prevent HOO-LnA-CmΔP generation. In addition, the possibility of the reaction between the linolenate allyl radical and free linolenate hydroperoxide should be negligible, because an excess amount of the nitroxyl radical competes with linolenate hydroperoxide for the linolenate allyl radical. Remarkably, the supplementation of linoleate hydroperoxide in the reaction solution promoted the one-electron redox reaction between α-linolenic acid and linolenate hydroperoxide catalyzed by lipoxygenase in the presence of CmΔP. It seems that the increase in coexisting hydroperoxides results in the acceleration of the lipohydroperoxidase reaction, in which the ferrous lipoxygenase is converted into the ferric one.

As supplementary data, we confirmed that removal of contaminant linolenate hydroperoxide from the α-linolenic acid solution used here did not influence HOO-LnA-CmΔP generation in the linolenate/lipoxygenase/CmΔP system.

In conclusion, we propose that a lipid peroxyl radical can be transformed into a hydroperoxy fatty acid allyl radical at lower oxygen content through intramolecular rearrangement. In the present study, we employed α-linolenic acid, because this PUFA possesses two doubly allylic methylenes at C-11 and C-14. In contrast, arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid possess more than three doubly allylic methylenes, so that the resulting hydroperoxy fatty acid allyl radicals show complexity in their structures. Subsequently, these intermediate carbon-centered radicals should give rise to several types of hydroperoxy fatty acids with structural diversity. In a future study, we plan to evaluate the real physiological functions of the hydroperoxy fatty acids produced in a PUFA/lipoxygenase system at lower oxygen content.

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