Cytochrome *c* catalyses the formation of pentyl radical and octanoic acid radical from linoleic acid hydroperoxide

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A reaction of 13-hydroperoxide octadecadienoic acid (13-HPODE) with cytochrome *c* was analysed using ESR, HPLC–ESR and HPLC–ESR–MS by the combined use of the spin-trapping technique. The ESR, HPLC–ESR and HPLC–ESR–MS analyses showed that cytochrome *c* catalyses formation of pentyl and octanoic acid radicals from 13-HPODE. On the other hand, only the α -(4-pyridyl-1-oxide)-*N*-t-butyl nitron/octanoic acid radical adduct was detected in the elution profile of HPLC–ESR for a mixture of 13-HPODE with haematin, indicating that

haematin catalyses the formation of octanoic acid radical. In addition, the reaction of 13-HPODE with cytochrome *c* was inhibited by chlorogenic acid, caffeic acid and ferulic acid via two possible mechanisms, i.e. reducing cytochrome *c* (chlorogenic acid and caffeic acid) and scavenging the radical intermediates (chlorogenic acid, caffeic acid and ferulic acid).

Key words: chlorogenic acid, free radical, HPLC–ESR, lipid peroxidation.

INTRODUCTION

Cytochrome *c* is an iron-containing haemoprotein that occurs in the respiratory chain. Inorganic and organic peroxides can instigate apoptosis, and cytochrome *c* can participate not only as a factor in caspase activation, as discovered recently, but also in propagating peroxidative damage. It has been shown that haemoproteins are catalysts for oxidations of lipids in various kinds of system, i.e. linoleic acid suspensions [1,2], mitochondrial membranes [3] and liposomes made from soya bean phospholipids [4]. The participation of several oxygen intermediates (O_2^- , $HO^\bullet$, H_2O_2 , singlet oxygen) and free-radical chain reactions is widely accepted as the mechanism of haemoprotein-catalysed lipid peroxidation. However, the participation of defined lipid-derived free-radical intermediates has not been determined. The haemoprotein-catalysed decomposition of lipid hydroperoxides has been studied extensively using ESR with the spin-trapping technique. The ESR spectra, which were observed in the reactions of peroxides with haemoproteins, were assigned to 5,5'-dimethyl-1-pyrroline *N*-oxide (DMPO)/peroxyl radical adducts, DMPO/alkoxyl radical adducts and DMPO/alkyl radical adducts [5–10]. On the other hand, a recent report on peroxide-derived radicals has shown that previously reported DMPO/peroxyl radical adducts are actually DMPO/alkoxyl radical adducts [11].

In this study, two kinds of lipid-derived free radical, pentyl radicals and octanoic acid radicals, which form in the reaction of 13-hydroperoxide octadecadienoic acid (13-HPODE) with cytochrome *c* or haematin, were separated and identified using HPLC–ESR spectrometry [12–15]. Furthermore, the effects of polyphenols, such as chlorogenic acid, caffeic acid and ferulic acid, on the formation of the respective lipid-derived free radicals are examined in the reaction mixture of 13-HPODE with cytochrome *c*.

MATERIALS AND METHODS

Materials

Linoleic acid (9,12-octadecadienoic acid), cytochrome *c* (horse heart) and soya bean lipoxigenase (EC 1.13.11.12) type V were

obtained from Sigma (St. Louis, MO, U.S.A.). Haemin was purchased from Nacalai Tesque (Kyoto, Japan). α -(4-Pyridyl 1-oxide)-*N*-t-butyl nitron (4-POBN), chlorogenic acid, caffeic acid, quinic acid and ferulic acid were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Ferrous ammonium sulphate was obtained from Kishida Chem. Co. (Osaka, Japan). EDTA was obtained from Wako Pure Chemicals (Osaka, Japan). Pentylhydrazine oxalate was synthesized according to the method of Gever and Hayes [16]. All other chemicals used were of analytical grade.

Preparation of 13-HPODE

The reaction mixture contained, in a total volume of 25 ml, 1.5 mg/ml linoleic acid, 440 units/ml soya bean lipoxigenase and 0.2 M boric acid (pH 9.0). The reaction was performed at 25 °C in air for 1 h. After the 1 h reaction, 0.4 ml of the reaction mixture was mixed with 3.6 ml of 0.2 M borate buffer (pH 9.0), and then injected into an HPLC with a UV detection system. The HPLC used consisted of a Rheodyne model 7125 injector (Rheodyne, Cotati, CA, U.S.A.), a Hitachi model 655A-11 pump with a model L-5000 LC controller (Hitachi, Ibaragi, Japan), a Waters μ Bondapak C_{18} semi-preparative column (30 mm $\times$ 10 mm inner diameter; Millipore, Milford, MA, U.S.A.), and an SPD-M10AVP diode array detector (Shimadzu Co., Kyoto, Japan) with a CLASS-LC10 LC workstation (Shimadzu). The SPD-M10AVP diode array detector was operated from 200 nm to 350 nm in the HPLC system. Two solvents were used in the HPLC: water (A) and methanol (B). A combination of isocratic and linear gradients was used for the HPLC: 0–20 min, 0–90 % solvent B (linear gradient) at a flow rate of 2.0 ml/min; 20–30 min, 90 % solvent B (isocratic) at a flow rate of 2.0 ml/min. A prominent peak was observed at a retention time of 26 min when the HPLC profile was monitored at 234 nm. The peak fraction was collected. The methanol contained in the fraction was removed using a model CC-105 centrifugal concentrator with a TU-055 low-temperature trap (Tomy, Yokohama, Japan). The water solution of the fraction was used

Abbreviations used: 13-HPODE, 13-hydroperoxide octadecadienoic acid; 4-POBN, α -(4-pyridyl 1-oxide)-*N*-t-butyl nitron; DMPO, 5,5'-dimethyl-1-pyrroline *N*-oxide.

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Figure 1 ESR spectra of the reaction of cytochrome *c* with 13-HPODE

The reaction and ESR conditions were as described in the Materials and methods section. Complete reaction mixture (A) with 50 μM cytochrome *c*, (B) without 50 μM cytochrome *c* and (C) without 13-HPODE. The vertical arrow indicates the peak that was used for peak-height estimation (see Figures 2, 8, 9, and 12). Magnetic field was also fixed at the position indicated by the arrow.

as a stock solution of 13-HPODE. The concentration of 13-HPODE was determined from its absorbance at 234 nm ($\epsilon = 25600 \text{ cm}^{-1} \cdot \text{M}^{-1}$) [17].

Preparation of 13-HPODE using normal-phase HPLC

From the reaction mixture of linoleic acid with lipoxygenase, 13-HPODE was prepared using normal-phase HPLC [18]. The reaction mixture (2 ml) was acidified to pH 2.0 and extracted three times with 2 ml of diethyl ether. The extracts were evaporated under reduced pressure and analysed using normal-phase HPLC. The HPLC was performed on a Zorbax SIL column (25.0 cm $\times$ 9.4 mm) with an n-hexane/diethyl ether/acetic acid mixture (1000:150:1, by vol.) at 20 °C. The flow rate was 3 ml/min.

Complete reaction mixture of 13-HPODE with cytochrome *c* or haematin

The complete reaction mixture of 13-HPODE with cytochrome *c* or haematin contained 0.14 mM 13-HPODE, 0.05 mM cytochrome *c* (or 0.005 mM haematin), 0.1 M 4-POBN and 40 mM phosphate buffer (pH 7.4). The reactions were started by adding cytochrome *c* or haematin. The reactions were performed for 2 min at 25 °C. The concentration of cytochrome *c* was determined from its absorbance at 550 nm ($\epsilon = 21000 \text{ cm}^{-1} \cdot \text{M}^{-1}$) after reduction with sodium dithionite [19].

ESR measurements

The ESR spectra were obtained using a JES-FR30 free radical monitor (Jeol, Tokyo, Japan). Aqueous samples were aspirated into a Teflon tube centred in a microwave cavity. Operating conditions of the ESR spectrometer were as follows: power, 4 mW; modulation width, 0.1 mT; centre of magnetic field,

337.0 mT; sweep time, 4 min; sweep width, 10 mT; time constant, 0.3 s. Magnetic fields were calculated by the splitting of MnO ($\Delta H_{3-4} = 8.69 \text{ mT}$).

HPLC-ESR analysis

The HPLC used in the HPLC-ESR analysis consisted of a model 7125 injector (Reodyne) with a 5 ml sample loop, a model 655A-11 pump and a model L-5000 LC controller (Hitachi). A Waters $\mu\text{Bondapak C}_{18}$ semi-preparative column (30 mm $\times$ 10 mm inner diameter; Millipore) was used. The column was kept at 25 °C throughout the analyses. For the HPLC-ESR analyses, two solvents were used: solvent A, 50 mM ammonium acetate; solvent B, 50 mM ammonium acetate/acetonitrile (36:64, v/v). A combination of isocratic and linear gradients was used: 0–40 min, 100–0 % A (linear gradient) at a flow rate of 2.0 ml/min; 40–60 min, 0 % A (isocratic) at 2.0 ml/min. The eluent was introduced into the JES-FR30 free-radical monitor. The ESR spectrometer was connected to the HPLC via a Teflon tube, which passed through the centre 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 ESR peak (indicated by a vertical arrow in Figure 1) throughout the experiments.

Preparation of the authentic 4-POBN/octanoic acid radical adduct

The authentic 4-POBN/octanoic acid radical adduct was prepared by the reaction of 13-HPODE with ferrous ions. The reaction mixture contained, in a total volume of 4 ml, 140 μM 13-HPODE, 0.33 mM $\text{FeSO}_4(\text{NH}_4)_2\text{SO}_4$, 0.1 M 4-POBN and 38 mM phosphate buffer (pH 7.4). The reaction was started by adding $\text{FeSO}_4(\text{NH}_4)_2\text{SO}_4$. The reaction was performed for 2 min at 25 °C. In order to purify the 4-POBN/octanoic acid radical adduct, 4.0 ml of the reaction mixture was applied to HPLC-ESR. The peak fraction appearing at 30 min on the HPLC-ESR

Figure 2 Cytochrome *c*- and haematin-concentration dependence of ESR peak height

The reaction and ESR conditions were as described in the Materials and methods section except for the concentration of cytochrome *c* and haematin. Total volume of the reaction mixture was 0.3 ml. The peak heights were evaluated from the peak height of the third signal of the ESR spectrum (Figure 1, arrow). ○, Cytochrome *c*; ●, haematin.

elution profile was collected. Identification of the 4-POBN/octanoic acid radical adduct has been described in a previous paper [20].

Synthesis of pentyl radicals

Authentic pentyl radicals were synthesized by the decomposition of pentylhydrazine. The reaction mixture contained, in a total volume of 1 ml, 0.1 M 4-POBN, 2.5 mg of pentylhydrazine oxalate, 0.2 mM CuCl₂ and 45 mM carbonate buffer (pH 10.0). After nitrogen gas was bubbled through the reaction mixture without CuCl₂ for 5 min, the reaction was started by adding CuCl₂. The reaction was performed for 2 h at 25 °C. In order to purify the 4-POBN/pentyl radical adduct, 0.5 ml of the pentyl radical reaction mixture was mixed with 4.5 ml of 50 mM ammonium acetate and then applied to the HPLC-ESR. The peak fraction that appeared at 47 min on the HPLC-ESR elution profile was collected. Identification of the 4-POBN/pentyl radical adduct has been described in a previous paper [20].

Visible absorption spectra

Visible absorption spectra were measured using a model UV-160A ultraviolet/visible spectrophotometer (Shimadzu). The spectrophotometer was operated from 450 to 650 nm. The measurements were performed at 25 °C. The reference cell contained 50 mM phosphate buffer (pH 7.4).

HPLC-ESR-MS analysis

The HPLC-ESR-MS apparatus consisted of a model 7125 Reodyne injector, a model L-7100 pump (Hitachi), a Waters μBondapak C₁₈ semi-preparative column (30 mm × 10 mm inner diameter; Millipore), a JES-FR30 free-radical monitor (Jeol) and a model M-1200AP LC-MS system with electrospray ionization (Hitachi). The HPLC and ESR conditions in the HPLC-ESR-MS analysis were the same as those in the HPLC-ESR analysis.

Figure 3 HPLC-ESR analysis of the reaction of cytochrome *c* with 13-HPODE

The reaction and ESR conditions were as described in the Materials and methods section. The reaction mixture (4.0 ml) was applied to HPLC-ESR. The y-axis shows an arbitrary scale. Complete reaction mixture (A) with cytochrome *c*, (B) without cytochrome *c* and (C) without 13-HPODE.

The operating conditions of the mass spectrometer were: nebulizer, 180 °C; aperture 1, 120 °C; N₂ controller pressure, 0.196 MPa; drift voltage, 70 V; multiplier voltage, 1800 V; needle voltage, 3000 V; polarity, positive; resolution, 48.

The complete reaction mixture of 13-HPODE with cytochrome *c* or haematin was applied to the HPLC-ESR-MS. The mass spectra of peaks 1 and 2 were obtained by introducing the eluent from the ESR detector into the LC-MS system just before the respective peaks were eluted. The flow rate was kept at 50 µl/min while the eluent was introduced into the LC-MS system.

RESULTS

ESR measurements of the reaction mixtures of 13-HPODE with cytochrome *c* or haematin

The ESR spectra of the complete reaction mixture of 13-HPODE with cytochrome *c*, the complete reaction mixture without 13-HPODE and the complete reaction mixture without cytochrome *c* were measured (Figure 1). A prominent ESR spectrum ($a^N = 1.58$ mT and $a^H\beta = 0.26$ mT) was observed in the complete reaction mixture with 50 µM cytochrome *c* (Figure 1A), which was hardly observed in the absence of 50 µM cytochrome *c* or 13-HPODE (Figures 1B and 1C). On the other hand, a prominent ESR spectrum ($a^N = 1.58$ mT and $a^H\beta = 0.26$ mT) was also observed in complete reaction mixture containing 13-HPODE and 5 µM haematin (results not shown). The ESR spectrum was hardly observed in the absence of 5 µM haematin or 13-HPODE (results not shown).

The ESR spectra of the complete reaction mixture of 13-HPODE with either cytochrome *c* or haematin were measured at various cytochrome *c* or haematin concentrations (Figure 2). ESR peak height increased with an increase in the concentration of cytochrome *c*. Conversely, ESR peak height for the haematin reaction mixture reached a maximum at 5 µM and then decreased gradually as haematin concentration increased.

Reaction-time dependence of ESR peak height was also examined for the complete reaction mixture of 13-HPODE with 50 µM cytochrome *c*. ESR peak height remained unchanged for 60 min (results not shown), indicating that the 4-POBN/13-HPODE-derived radical adducts were relatively stable in this reaction mixture.

Identification of the radicals formed in the reaction mixture of 13-HPODE with cytochrome *c*

HPLC-ESR analyses were performed for the complete reaction mixture, the complete reaction mixture without cytochrome *c* and the complete reaction mixture without 13-HPODE (Figure 3). On the HPLC-ESR elution profile of the complete reaction mixture, two prominent peaks were observed at the retention times of 30.2 ± 0.5 min (peak 1) and 47.2 ± 0.3 min (peak 2; Figure 3A). Peaks 1 and 2 were detected with a peak-height ratio of 4.3:1.0. These peaks were not observed on the elution profiles of the complete reaction mixture without cytochrome *c* or 13-HPODE (Figures 3B and 3C). Since 9-hydroperoxide octadecadienoic acid may be contained in the 13-HPODE fraction obtained by reversed-phase HPLC, 13-HPODE was purified using normal-phase HPLC [18]. Four separate peaks (P53.4, P60.5, P64.8 and P66.7) were detected in the normal-phase HPLC elution profile at the retention times of 53.4, 60.5, 64.8 and 66.7 min. Based on the study by Teng and Smith [18], we assigned the four peaks as follows: P53.4, 13-hydroperoxy-(9Z,11E)-octadeca-9,11-dienoic acid; P60.5, 13-hydroperoxy-(9E,11E)-octadeca-9,11-dienoic acid; P64.8, 9-hydroperoxy-(10E,12Z)-octadeca-10,12-dienoic acid; P66.7,

Figure 4 HPLC-ESR analysis of the complete reaction mixture with cytochrome *c*, the authentic 4-POBN/pentyl radical adduct and the authentic 4-POBN/octanoic acid radical adduct

The reaction and HPLC-ESR conditions were as described in the Materials and methods section. (A) Complete reaction mixture (4.0 ml) with cytochrome *c*. (B) Authentic 4-POBN/pentyl radical adduct solution (15 µl) was mixed with 3.985 ml of 50 mM ammonium acetate and applied to HPLC-ESR. (C) Complete reaction mixture (3.985 ml) with cytochrome *c* was mixed with 15 µl of authentic 4-POBN/pentyl radical adduct solution and applied to HPLC-ESR. (D) Authentic 4-POBN/octanoic acid radical adduct solution (0.15 ml) was mixed with 3.85 ml of 50 mM ammonium acetate and applied to HPLC-ESR analysis. Amplification of ESR was four times that of (A), (B) and (C). (E) Complete reaction mixture with cytochrome *c* (0.6 ml), 0.3 ml of authentic 4-POBN/octanoic acid radical adduct solution and 3.1 ml of 50 mM ammonium acetate were mixed and applied to HPLC-ESR. Amplification of ESR was twice that of (A), (B) and (C).

9-hydroperoxy-(10E,12E)-octadeca-10,12-dienoic acid. Relative intensities (at 234 nm) of the four peaks were as follows: P53.4, 87.7%; P60.5, 1.4%; P64.8, 6.2%; P66.7, 4.7%. We collected P53.4 and used it for the reaction. In addition to the four peaks, a small peak was observed at the retention time of 70.9 min. The intensity of this peak was less than half of that for P60.5.

When the 13-HPODE obtained by normal-phase HPLC was used for the reaction, peaks 1 and 2 were also detected, with the same peak-height ratio as for reversed-phase HPLC, suggesting that the 13-HPODE purified by reversed-phase HPLC was adequately pure for this experiment.

In order to identify the peak 1 and peak 2 compounds, 15 µl of authentic 4-POBN/pentyl radical adduct solution was mixed with 3.985 ml of 50 mM ammonium acetate and then applied to HPLC-ESR analysis (Figure 4B). The 4-POBN/pentyl radical adduct was eluted at the retention time of 46.9 min (Figure 4B).

Figure 5 HPLC-ESR-MS analyses of complete reaction mixture of cytochrome *c* with 13-HPODE

The reaction and HPLC-ESR-MS conditions were as described in the Materials and methods section. (A) Mass spectrum of peak 1; (B) mass spectrum of peak 2.

Figure 6 HPLC-ESR analyses of complete reaction mixture of haematin

The reaction and HPLC-ESR conditions were as described in the Materials and methods section. The complete reaction mixture (4.0 ml) of 50 μ M cytochrome *c* or 5 μ M haematin was applied to HPLC-ESR. The y-axis shows an arbitrary scale. (A) Cytochrome *c*; (B) haematin.

Figure 7 Structures of chlorogenic acid and related compounds

The retention time was almost the same as that of the peak 2 compound. When 3.985 ml of the complete reaction mixture of cytochrome *c* was mixed with 15 μ l of authentic 4-POBN/pentyl radical adduct solution and then applied to the HPLC-ESR, the peak height of the peak 2 compound increased (Figure 4C), indicating that peak 2 was the 4-POBN/pentyl radical adduct. HPLC-ESR-MS analysis of peak 2 gave ions at m/z 179 and m/z 266 (Figure 5B), supporting the above identification. The ion m/z 266 corresponded to the protonated molecular ion of the 4-POBN/pentyl radical adduct, $(M+H)^+$. A fragment ion at m/z 179 corresponded to the loss of $[(CH_3)_3C(O)N]$ from the protonated molecular ion.

Authentic 4-POBN/octanoic acid radical adduct solution (0.15 ml) was mixed with 3.85 ml of 50 mM ammonium acetate, and then applied to HPLC-ESR analysis. The 4-POBN/octanoic acid radical adduct was eluted at the retention time of 29.8 min (Figure 4D). The retention time was almost the same as for peak 1. The complete reaction mixture of cytochrome *c* (0.6 ml), the authentic 4-POBN/octanoic acid radical adduct solution (0.3 ml) and 50 mM ammonium acetate (3.1 ml) were mixed and then applied to HPLC-ESR. The height of peak 1 increased (Figure 4E), indicating that the peak 1 compound was the 4-POBN/octanoic acid radical adduct. The HPLC-ESR-MS analysis of peak 1 compound gave ions at m/z 251 and m/z 338 (Figure 5A), supporting the above identification. The ion m/z 338 corresponded to the protonated molecular ion of 4-POBN/octanoic acid radical adduct, $(M+H)^+$. A fragment ion at m/z 251 corresponded to the loss of $[(CH_3)_3C(O)N]$ from the protonated molecular ion.

HPLC-ESR and HPLC-ESR-MS analyses of the reaction mixture of 13-HPODE with haematin

To find out what kinds of radicals form in the reaction mixture of 13-HPODE with haematin, HPLC-ESR analyses of this reaction mixture were performed. A prominent peak was observed at the retention time of 29.7 min on the HPLC-ESR elution profile (Figure 6B). Based on the retention time, the peak can be assigned to 4-POBN/octanoic acid radical adduct. The

Figure 8 Effects of caffeic acid, ferulic acid and quinic acid on the formation of 13-HPODE-derived radicals

The ESR spectra were observed for the complete reaction mixture of cytochrome *c* with various concentrations of caffeic acid ($\circ$), quinic acid ($\bullet$) or ferulic acid ($\triangle$). Signal intensities were evaluated from the peak height of the third ESR signal of the 4-POBN/13-HPODE-derived radical adducts (Figure 1, arrow). The control value of 100% represents the level of 4-POBN/13-HPODE-derived radical adducts formed in the absence of any added compounds. ESR conditions were as described in the Materials and methods section.

HPLC-ESR-MS analysis of the peak gave ions at m/z 251 and m/z 338, supporting the above identification. The ion m/z 338 corresponded to the protonated molecular ion of the 4-POBN/octanoic acid radical adduct, $(M+H)^+$. A fragment ion at m/z 251 corresponds to the loss of $[(CH_3)_3C(O)N]$ from the protonated molecular ion. A peak corresponding to the 4-POBN/pentyl radical adduct (Figure 6A) was not detected on the HPLC-ESR elution profile of haematin. When 13-HPODE that had been purified by normal-phase HPLC was used for the reaction of haematin, a similar HPLC-ESR elution profile was observed.

Inhibitory effect of polyphenols on the formation of 13-HPODE-derived radicals

To examine the inhibitory effect of polyphenols, such as chlorogenic acid, caffeic acid, ferulic acid and quinic acid (Figure 7), on the formation of 13-HPODE-derived radicals, various concentrations of chlorogenic acid (or caffeic acid, ferulic acid or quinic acid) were added to the complete reaction mixture of cytochrome *c* (Figures 7 and 8). Chlorogenic acid, which is an ester formed from caffeic acid and quinic acid, inhibited the formation of 13-HPODE-derived radicals, but quinic acid itself did not. The inhibitory effect seems to have been due to the caffeic acid moiety in chlorogenic acid. Indeed, caffeic acid inhibited the formation of 13-HPODE-derived radicals.

Since the inhibitory effect of chlorogenic acid on the ferrous ion-catalysed 13-HPODE-derived radical formation was diminished in the presence of EDTA [18], EDTA was added to the complete reaction mixture of cytochrome *c*. Chlorogenic acid showed the inhibitory effect on the formation of 13-HPODE-derived radicals, even in the presence of EDTA (Figure 9), indicating that chlorogenic acid exerts an inhibitory effect on the formation of 13-HPODE-derived radicals in the reaction mixtures of ferrous ions and cytochrome *c* through different mechanisms.

Figure 9 Effects of chlorogenic acid on the formation of 13-HPODE-derived radicals in the presence of EDTA

The ESR spectra were observed for the complete reaction mixture of cytochrome *c* with various concentrations of chlorogenic acid in the presence (●) or absence (○) of 1 mM EDTA. Signal intensities were evaluated from the peak height of the third ESR signal of the 4-POBN/13-HPODE-derived radical adducts (Figure 1, arrow). The control value of 100% represents the level of 4-POBN/13-HPODE-derived radical adducts formed in the absence of chlorogenic acid. ESR conditions were as described in the Materials and methods section.

Figure 10 Effects of chlorogenic acid on the formation of octanoic acid and pentyl radicals

Reaction and HPLC-ESR conditions were as described in the Materials and methods section. Reaction mixture (4.0 ml) was applied to the HPLC-ESR. Complete reaction mixture with 50 μ M cytochrome *c* in the absence (A) or presence (B) of 50 μ M chlorogenic acid.

In order to examine the effect of chlorogenic acid on the formation of the respective 13-HPODE-derived radicals, the HPLC-ESR analyses were performed for the complete reaction mixture of cytochrome *c* (Figure 10). On addition of chlorogenic acid, peak heights of the 4-POBN/octanoic acid radical adduct (peak 1) and 4-POBN/pentyl radical adduct (peak 2) decreased to 26% and 33% of the control, respectively (Figure 10B).

The visible absorption spectra of cytochrome *c* with caffeic acid

To examine the inhibitory effect of caffeic acid on the formation of 13-HPODE-derived radicals, the visible absorption spectra of

Figure 11 Visible spectra of cytochrome *c* with or without caffeic acid

The visible spectra were observed for cytochrome *c* with (solid line) or without (dotted line) 200 μ M caffeic acid in 50 mM phosphate buffer (pH 7.4).

Figure 12 Effects of KCN on the formation of 13-HPODE-derived radicals

The ESR spectra were observed for the complete reaction mixture of cytochrome *c* with various concentrations of KCN. Signal intensities were evaluated from the peak height of the third ESR signal of the 4-POBN/13-HPODE-derived radical adducts (Figure 1, arrow). The control value of 100% represents the level of 4-POBN/13-HPODE-derived radical adducts formed in the absence of KCN. Reaction and ESR conditions were as described in the Materials and methods section.

cytochrome *c* with or without caffeic acid were measured (Figure 11). The visible spectrum of cytochrome *c* showed a band with a $\lambda_{\max}$ value of 529 nm. On addition of caffeic acid, new visible bands with $\lambda_{\max}$ values of 550 and 521 nm appeared, indicating that caffeic acid reduces cytochrome *c*.

Effect of CN⁻ ions on the formation of 13-HPODE-derived radicals

To examine the effects of KCN on the formation of 13-HPODE-derived radicals, various concentrations of KCN were added to the control reaction mixture of cytochrome *c* (Figure 12). Depending on the dose, KCN inhibited the formation of 13-HPODE-derived radicals.

DISCUSSION

In this study, the 4-POBN/octanoic acid and 4-POBN/pentyl radical adducts were detected and identified in the reaction mixture of 13-HPODE with cytochrome *c* or haematin using HPLC-ESR and HPLC-ESR-MS. Our previous studies have also shown the formation of the octanoic acid and pentyl radicals

in the reaction mixtures of linoleic acid with soya bean lipoxygenase and 13-HPODE with ferrous ions [12,15,20]. Possible reaction paths for the formation of these radicals are shown in Scheme 1. One-electron reductive homolytic decomposition of the lipid hydroperoxide (LOOH) by cytochrome *c* (cyt *c*) results in the formation of alkoxy radicals [10,21,22]:

Scheme 1 Proposed mechanism for the formation of pentyl and octanoic acid radicals in the reaction of 13-HPODE with cytochrome *c*

Barr and Mason [23] and Rota et al. [10] have also presented homolytic scission for the reaction of cytochrome *c* with *t*-butylhydroperoxide [23] and the reaction of cytochrome P450 with 13-HPODE [10]. On the other hand, it is also possible for heterolytic peroxide bond cleavage to result in the formation of the alkoxyl radical, as follows [24–26]:

Product analysis and spin-trapping studies provided evidence for the formation of 13-alkoxylinoic acid radicals through the reaction of 13-HPODE with ferrous ions [6,27,28]. The β -scission of 13-alkoxylinoic acid radicals results in the formation of pentyl radicals [12,13,15,29]. The pentyl radical could be a precursor of pentane. Garssen et al. [30] have reported the formation of pentane and 3-oxo-9,11-tridecadienoic acid in the reaction mixture of soya bean lipoxigenase with linoleic acid. On the other hand, the 13-alkoxylinoic acid radical possibly rearranges intramolecularly by the addition of a double bond to cause the formation of the 12,13-epoxylinoic acid radical. Indeed, the 12,13-epoxylinoic acid radical was detected and identified in the reaction mixture of soya bean lipoxigenase with linoleic acid in borate buffer (pH 9.0), using 2-methyl-2-nitrosopropane as a spin-trapping reagent [13]. The reaction of the 12,13-epoxylinoic acid radical with an oxygen molecule results in the formation of the 12,13-epoxy-9-hydroperoxylinoic acid radical. Gardner et al. [31] have reported that 12,13-epoxy-9-hydroperoxylinoic acid forms in the reaction mixture of 13-HPODE with cysteine/FeCl₃. It is possible that the octanoic acid radical (peak 1) forms through β -scission of the 12,13-epoxy-9-alkoxylinoic acid radical. Although the 12,13-epoxylinoic acid radical is a putative intermediate in this reaction, it was not trapped. The reason for this lies with the spin-trapping reagent used, 4-POBN. Indeed, the 12,13-epoxylinoic acid radical was not detected as a main product in the reaction mixture of soya bean lipoxigenase with linoleic acid using 4-POBN as a spin-trapping reagent [15], although this radical was detected using 2-methyl-2-nitrosopropane as a spin-trapping reagent [13].

Octanoic acid radicals and pentyl radicals form with a peak-height ratio of 1.0:4.3 in the cytochrome *c* reaction mixture. On the other hand, only the octanoic acid radical forms in the reaction of 13-HPODE with haematin (Figure 3). Haematin and cytochrome *c* may catalyse the formation of the 13-HPODE-derived radicals through quite different mechanisms. Apoprotein of cytochrome *c* may play an important role in the reaction. On the other hand, less oxygen consumption due to weak catalytic activity of haematin may result in the predominant formation of the octanoic acid radical in the haematin reaction mixture, since the reaction of a 12,13-epoxylinoic acid radical with an oxygen molecule forms a 12,13-epoxy-9-hydroperoxylinoic acid radical (Scheme 1).

Polyphenols such as caffeic acid, chlorogenic acid and ferulic acid inhibited the formation of octanoic acid radicals and pentyl radicals in the reaction mixture of 13-HPODE with cytochrome *c* (Figures 7 and 8). On examining the inhibitory effects of chlorogenic acid and caffeic acid at different concentrations, it seems that they inhibit the formation of pentyl and octanoic acid radicals in two ways. One of the mechanisms acts predominantly at a low inhibitor concentration and the other is exerted at a high inhibitor concentration. Caffeic acid and chlorogenic acid seem to inhibit by reducing cytochrome *c* at relatively low concentrations (Figure 11). On the other hand, caffeic acid and

chlorogenic acid also appear to inhibit the formation of pentyl and octanoic acid radicals by scavenging radical intermediates at high concentrations. Ferulic acid seems to inhibit the formation of pentyl and octanoic acid radicals only by scavenging radical intermediates.

KCN may exert an inhibitory effect on the formation of pentyl and octanoic acid radicals through co-ordination with haem. As shown in Figure 12, addition of 6.2 mM KCN to the reaction mixture containing 50 μM cytochrome *c* resulted in 50 % inhibition. Assuming that free cytochrome *c* catalyses the formation of pentyl and octanoic acid radicals, 2.5 μM free cytochrome *c* exists in the solution containing 50 μM cytochrome *c* and 6.2 mM KCN (see Figure 2). Thus an association constant between cytochrome *c* and CN[−] of $3.1 \times 10^3 \text{ M}^{-1}$ can be estimated, which is comparable with the previously reported value of $5.3 \times 10^3 \text{ M}^{-1}$ [32].

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Received 26 April 2001/14 September 2001; accepted 24 October 2001