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Identification of a radical formed in the reaction mixture of rat brain homogenate with a ferrous ion/ascorbic acid system using HPLC–EPR and HPLC–EPR–MS

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Abstract

Identification of a free radical is performed for the reaction mixture of rat brain homogenate with a ferrous ion/ascorbic acid system using EPR, high performance liquid chromatography–electron paramagnetic resonance spectrometry (HPLC–EPR) and high performance liquid chromatography–electron paramagnetic resonance–mass spectrometry (HPLC–EPR–MS). EPR measurements of the reaction mixtures showed prominent signals with hyperfine coupling constants ($\alpha^{N} = 1.58$ mT and $\alpha^{H}\beta = 0.26$ mT). No EPR spectrum was detectable without rat brain homogenate, suggesting that the radical is derived from rat brain homogenate. An HPLC–EPR analysis of the reaction mixture showed a peak with retention time of 33.7 min. An HPLC–EPR–MS analysis of the peak gave two ions at *m*/*z* 224 and 137, suggesting that α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN)/ethyl radical adduct forms in the reaction mixture.

Keywords: *Lipid peroxidation, HPLC–EPR, HPLC–EPR–MS, ethyl radical, rat brain, spin trapping*

Introduction

Brain tissue is rich in polyunsaturated fatty acids. These polyunsaturated fatty acids are especially vulnerable to oxidation [1]. Brain injury has been related to numerous mechanisms, among which the generation of free radicals has attracted special attention [2–5].

Indeed, the semi-dehydroascorbate radical levels increased in the hypoxic ischemic hemisphere [6], in the rat brain subjected to traumatic brain injury [7,8] and in mouse hippocampal slices during and after kainate-induced seizures [9]. Hydroxyl radicals and carbon-centered radicals were detected when cells, derived from rat hypothalamus, were incubated with 100 μM Fe–dopamine complex in the presence of 100 μM dicoumarol [10]. Hydroxyl radicals was also detected after pentylenetetrazole-induced seizure [11]. Carbon-centered radicals were observed in the rat brain subjected to traumatic brain injury, cerebral ischemia damage and kainic acid-induced seizures [7,12,13]. Alkoxyl and nitric oxide free radicals were detected during in utero hypoxia in a fetal guinea pig brain [14–16].

In spite of the intensive electron paramagnetic resonance (EPR) studies, many radical species have not been identified. In this study, identification of the free radicals is performed for the reaction mixture of rat brain homogenate with a ferrous ion/ascorbic acid

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system using EPR, HPLC–EPR and HPLC–EPR–MS [17].

Materials and methods

Materials

α-(4-Pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN) was purchased from Tokyo Kasei Kogyo, Ltd. (Tokyo, Japan). Ferrous ammonium sulfate was obtained from Kishida Chem. Co. (Osaka, Japan). Ethylenediaminetetraacetic acid disodium salt (EDTA) and ascorbic acid were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 1,2-Diethylhydrazine dihydrochloride was obtained from Aldrich Chem. Co. (Milwaukee, WI, USA). Sep-pak C_{18} was from Waters Associates (Milford, MA, USA). All other chemicals used were of analytical grade.

Preparation of 4-POBN/ethyl radical adduct

4-POBN/ethyl radical adduct was synthesized through the decomposition of 1,2-diethylhydrazine [14]. The reaction mixture contained 0.1 M 4-POBN, 2.5 mg/ml 1,2-diethylhydrazine dihydrochloride, 0.2 mM $CuCl_2$ and 44 mM carbonate buffer (pH 10.0). After nitrogen gas was bubbled through the reaction mixture without $CuCl_2$ for 5 min, the reaction was started by adding $CuCl_2$. The reaction was performed for 2 h at 25°C.

Preparation of rat brain homogenate

Wister rats (male, 308–317 g body weight) were decapitated. Whole rat brain was removed and homogenized in ice-cold 50 mM phosphate buffer pH 7.4 (1:10, w/v).

The reaction mixture of rat brain homogenate with a ferrous ion/ascorbic acid system

The reaction mixture contained 1 g/75 ml rat brain homogenate, 0.1 M 4-POBN, 7.5 μM $FeSO_4(NH_4)_2SO_4$, 0.15 mM ascorbic acid, 7.5 μM EDTA and 39.3 mM phosphate buffer (pH 7.4). The reaction was started by adding $FeSO_4(NH_4)_2SO_4$. After 300 min reaction at 25°C, the reaction mixtures were applied to EPR, HPLC–EPR and HPLC–EPR–MS.

EPR measurements

The EPR 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 EPR 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 ($\Delta H_{3-4} = 8.69$ mT).

HPLC–EPR chromatography

An HPLC used in the HPLC–EPR 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 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–EPR experiments. For the HPLC–EPR, two solvents were used: solvent A, 50 mM ammonium acetate; solvent B, 50 mM ammonium acetate/acetonitrile (36:64, v/v). A following combination of isocratic and linear gradient was used: 0–40 min, 100–0% A (linear gradient); 40–60 min, 100% B (isocratic). The eluent was introduced into a model JES-FR30 Free Radical Monitor (JEOL Ltd. Tokyo, Japan). The EPR spectrometer was connected to the HPLC with a Teflon tube, which passed through the center of the EPR cavity. The operating conditions of the EPR 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 EPR spectrum ($\alpha^N = 1.58$ mT and $\alpha^H\beta = 0.26$ mT) of the 4-POBN radical adduct (Figure 1).

HPLC–EPR–MS chromatography

HPLC and EPR conditions were as described in the HPLC–EPR. The operating conditions of the mass spectrometer were: nebulizer, 180°C; aperture 1, 120°C; N_2 controller pressure, 19.6 N/cm^2; drift

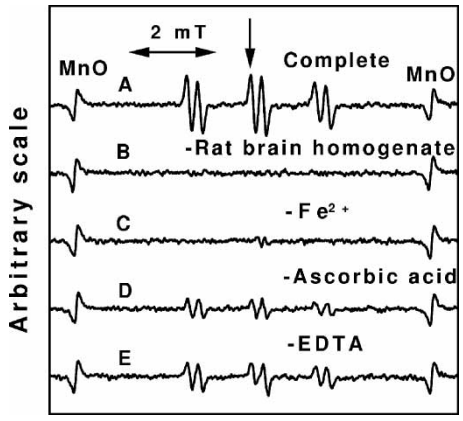


Figure 1. EPR spectra of the reaction mixtures of rat brain homogenate with a ferrous ion/ascorbic acid system. The reaction and EPR conditions were as described under "Materials and methods". The reaction time is 5 h. (A) A complete reaction mixture of rat brain homogenate with a ferrous ion/ascorbic acid system. (B) Same as in A except that rat brain homogenate was omitted. (C) Same as in A except that ferrous ions were omitted. (D) Same as in A except that ascorbic acid was omitted. (E) Same as in A except that EDTA was omitted.

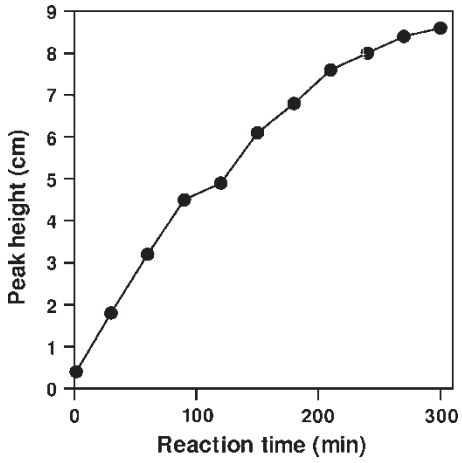


Figure 2. Time course of the EPR peak height of the complete reaction mixture. The reaction and EPR conditions were as described under "Materials and methods".

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 EPR detector into the LC-MS system just before the peak was eluted. The flow rate was kept at 50 μl/min while the eluent was introducing into the mass spectrometer.

Results and discussion

EPR measurements of the reaction mixture of rat brain homogenate with a ferrous ion/ascorbic acid system

EPR spectrum of the complete reaction mixture of rat brain homogenate with a ferrous ion/ascorbic acid system was measured (Figure 1). A prominent EPR spectrum ($\alpha^{N} = 1.58$ mT and $\alpha^{H}\beta = 0.26$ mT) was observed in the complete reaction mixture. The EPR spectrum was hardly observed for the complete reaction mixture without rat brain homogenate (or ferrous ion) (Figure 1(B) (C)). The EPR peak height of the reaction mixture without ascorbic acid (or EDTA) decreased to 35% (or 54%) of the complete reaction mixture (Figure 1(D) and (E)).

Time course of the EPR peak height was measured for the complete reaction mixture (Figure 2). The EPR peak height increased with time for 5 h.

HPLC–EPR and HPLC–EPR–MS analyses of the reaction mixture of rat brain homogenate with a ferrous ion/ascorbic acid system

To identify the radicals formed in the complete reaction mixture of the rat brain homogenate with a ferrous ion/ascorbic acid system, HPLC–EPR analyses were performed. Four prominent peaks (peaks 1, 2, 3 and 4) were separated on the HPLC–EPR elution profile of the complete reaction mixture of the rat brain homogenate with a ferrous ion/ascorbic acid system (Figure 3(A)).

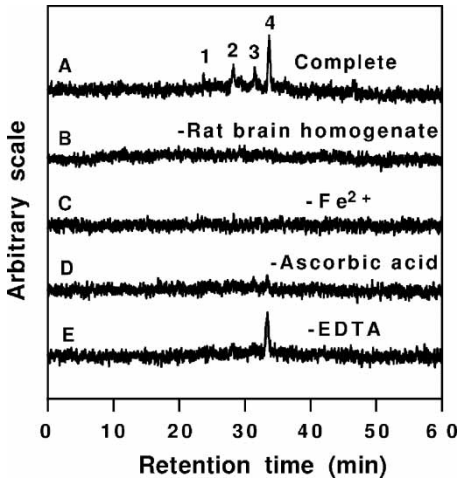


Figure 3. HPLC–EPR analyses of the reaction mixtures of rat brain homogenate with a ferrous ion/ascorbic acid system. The reaction and HPLC–EPR conditions were as described under "Materials and methods". Total volume of the reaction mixtures was 9.0 ml. (A) A complete reaction mixture of rat brain homogenate with a ferrous ion/ascorbic acid system. (B) Same as in A except that rat brain homogenate was omitted. (C) Same as in A except that ferrous ions were omitted. (D) Same as in A except that ascorbic acid was omitted. (E) Same as in A except that EDTA was omitted.

The retention times of the peaks 1, 2, 3 and 4 are as follows: peak1, 25.4 min; peak 2, 28.3 min; peak 3, 31.5 min; peak 4, 33.7 min, respectively. The peaks were hardly observed for the complete reaction mixture without rat brain homogenate, suggesting that these radicals are derived from rat brain homogenate (Figure 3(B)). Iron ion is essential for the reaction because the peaks were not observed for the complete reaction mixture without iron ion (Figure 3(C)). Peak height of the respective peaks decreased for the complete reaction mixture in the absence of ascorbic acid (Figure 3(D)). The ascorbic acid may enhance the formation of the radicals by reducing iron ion and/or oxygen molecule in the reaction mixture [18]. Peak height also decreased for the complete reaction mixture in the absence of EDTA (Figure 3(E)). In the complete reaction mixture, the EDTA could enhance the formation of the radicals by chelating of the iron ion [19–21].

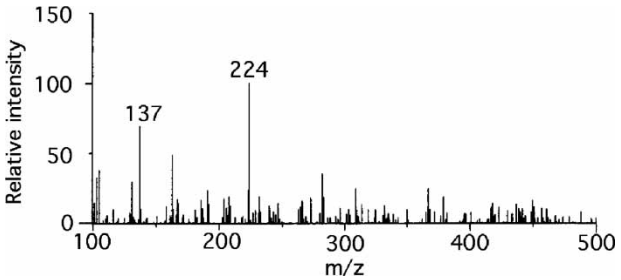


Figure 4. HPLC–EPR–MS analysis of the reaction mixture of rat brain homogenate with a ferrous ion/ascorbic acid system. The reaction and HPLC–EPR–MS conditions were as described under "Materials and methods". Total volume of the reaction mixture was 9.0 ml.

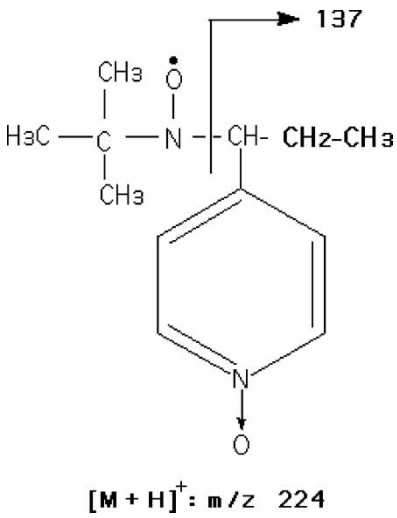


Figure 5. A possible structure of the peak 4.

To determine the structure of the peak 4, HPLC–EPR–MS analysis was performed. HPLC–EPR–MS analysis of the peak 4 compound gave ions at *m/z* 224 and 137 (Figure 4). The ion *m/z* 224 corresponds to the protonated molecules of 4-POBN/ethyl radical adduct, $[M + H]^+$. A fragment ion at *m/z* 137 corresponds to the loss of $(CH_3)_3C(O)N$ from the protonated molecules (Figure 5). For the three peaks (peaks 1, 2 and 3), HPLC–EPR–MS analyses did not succeed because of weak intensities of the peaks.

In order to confirm the structure of the peak, HPLC–EPR analysis was performed. An HPLC–EPR analysis of the reaction mixture of 1,2-diethylhydrazine with Cu^{2+} showed almost the same retention time as the peak 4 (Figure 6(B)). When the peak fraction of the reaction mixture of 1,2-diethylhydrazine with Cu^{2+} was mixed with the reaction mixture of rat brain homogenate with a ferrous ion/ascorbic acid system, the peak height of peak 4 increased (Figure 6(C)), suggesting that the peak 4 and 4-POBN/ethyl radical adduct are identical.

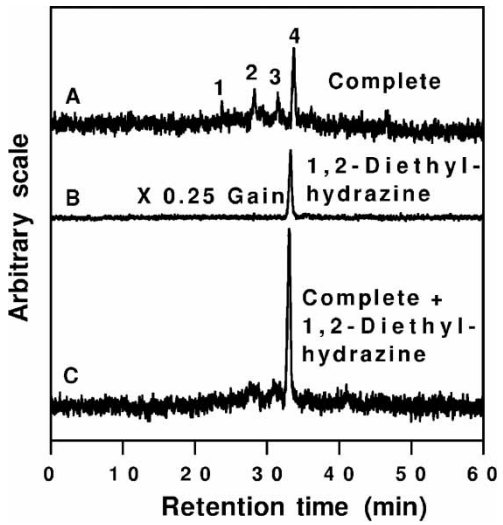


Figure 6. HPLC–EPR analyses of the reaction mixtures of rat brain homogenate with a ferrous ion/ascorbic acid system and of 1,2-diethylhydrazine with Cu^{2+}. The HPLC–EPR and reaction conditions were as described under "Materials and methods". (A) Complete reaction mixture of rat brain homogenate with a ferrous ion/ascorbic acid system (9 ml). (B) Reaction mixture of 1,2-diethylhydrazine with Cu^{2+} (0.3 ml). (C) A mixture of A (9 ml) and B (0.075 ml).

Possible reaction paths for the formation of the ethyl radical are shown in Scheme 1. EDTA-Fe(III) is reduced by ascorbate, which generated the ferryl ion EDTA-Fe(IV) = O by "pumping" electrons [22,23]. Reaction of EDTA-Fe(IV) = O with unsaturated fatty acid LH such as eicosapentaenoic acid results in the formation of allyl radical $L^{\cdot}$ (equation (1)).

$$\text{EDTA–Fe(IV)} = \text{O} + \text{LH} \rightarrow \text{EDTA–Fe(III)} + \text{L}^{\cdot} + \text{HO}^{-} \quad (1)$$

Peroxyl radical $LOO^{\cdot}$ is likely to form through the reaction of the allyl radical $L^{\cdot}$ with molecular oxygen O_2 (equation (2)).

$$\text{L}^{\cdot} + \text{O}_2 \rightarrow \text{LOO}^{\cdot} \quad (2)$$

It seems plausible that the peroxyl radical $LOO^{\cdot}$ turns to hydroperoxides LOOH by removing $H^{\cdot}$ from surrounding molecules (equation (3)).

$$\text{LOO}^{\cdot} + \text{H}^{\cdot} \rightarrow \text{LOOH} \quad (3)$$

EDTA-Fe^{2+} possibly catalyzes decomposition of hydroperoxides LOOH [16]. This reaction yields radical intermediates $LO^{\cdot}$ (equation (4)) [17].

$$\text{EDTA–Fe}^{2+} + \text{LOOH} \rightarrow \text{EDTA–Fe}^{3+} + \text{LO}^{\cdot} + \text{OH}^{-} \quad (4)$$

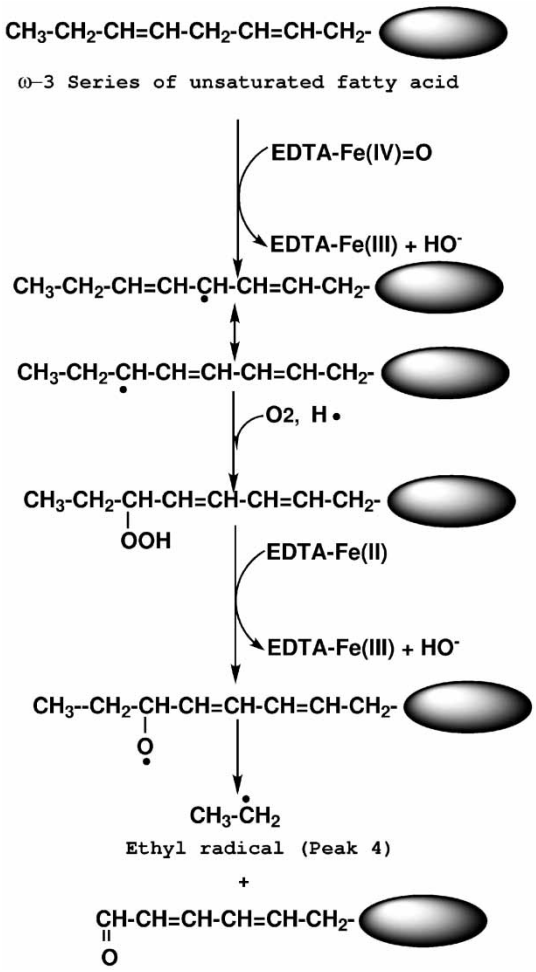
β-Scission of the $LO^{\cdot}$ could form the ethyl radical (equation (5)). Thus, the ethyl radical may form through oxidation of ω-3 series of unsaturated fatty acids.

$$\text{LO}^{\cdot} \rightarrow \text{Ethyl radical} \quad (5)$$

Several kinds of radical species such as $L^{\cdot}$ and $LO^{\cdot}$ must form during the reaction. However, these radicals were not detected in this study. The reason we failed to detect these radicals may be that the 4-POBN/$L^{\cdot}$(or $LO^{\cdot}$) radical adducts are unstable or reaction rate of 4-POBN with the radicals ($L^{\cdot}$ and $LO^{\cdot}$) is too slow.

To our knowledge the ethyl radical was first identified in the rat brain subjected to an oxidative stress. The ethyl radical is an important biomarker to clarify the mechanism of oxidative stress in the body.

The measurement of ethane in expired air has offered a noninvasive means to assess *in vivo* and *in vitro* lipid peroxidation [24–26]. The ethyl radical detected in this paper could be proposed to form ethane by abstracting a hydrogen atom from the surrounding molecules.



Scheme 1. Possible reaction paths for the formation of the ethyl radical.

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