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DETECTION OF THE ETHYL- AND PENTYL-RADICAL ADDUCTS OF α-(4-PYRIDYL-1-OXIDE)-*N-TERT*-BUTYLNITRONE IN RAT-LIVER MICROSOMES TREATED WITH ADP, NADPH AND FERRIC CHLORIDE

HIDEO IWAHASHI†, CAROL E. PARKER, KENNETH B. TOMER and RONALD P. MASON

Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, USA

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HPLC-EPR analyses of the reaction mixtures of microsomal suspensions incubated with ADP, ferric chloride, NADPH and α-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone (4-POBN) were performed. In the elution pattern of the reaction mixture, three peaks (peaks 1, 2 and 3) were detected. The radical adducts (1 and 3) were identified as being the pentyl- and ethyl-radical adducts of 4-POBN by comparing their retention times with those of the authentic radical adducts.

KEY WORDS: ethyl radical, pentyl radical, spin trapping, microsomal oxidation, HPLC-EPR.

INTRODUCTION

Lipid peroxidation has attracted considerable interest because of its supposed role in cancer, inflammation and tissue injury. Radicals have been detected in both *in vivo* and *in vitro* lipid peroxidation systems. For example, EPR techniques have been used to study free-radical generation during carbon tetrachloride poisoning[1–10] and following the exposure of hepatocytes and subcellular fractions to ethanol[11–14] and iron.[15–18] EPR experiments employing [^{13}C]-labelled compounds[2,3,5–8,11,12] and mass spectral determinations[9,10] have allowed the identification of the carbon tetrachloride- and ethanol-derived radicals ($\cdot CCl_3$, and $\cdot CH(OH)CH_3$) in such systems. In addition, lipid-derived radicals have been detected. Using deuterated α-phenyl-*N-tert*-butyl nitrones,[7,9,10] Janzen *et al.* have demonstrated that the lipid-derived radicals are of the primary alkyl ($\cdot CH_2R$) and the alkoxyl types ($\cdot OL$). These chemical structures of the radical adducts, however, have not been determined unambiguously.

In this study, in order to identify the above lipid-derived radicals in more detail, we have attempted to analyse the products of ADP/Fe^{3+}-stimulated microsomal lipid peroxidation using HPLC-EPR techniques.[19–22] ADP/Fe^{3+} has been shown to stimulate greater levels of lipid peroxidation than carbon tetrachloride.[15] It is shown that the HPLC retention time, as determined using EPR detection, provides valuable

† *Present address and address for correspondence*: Department of Chemistry, Wakayama Medical College, 651 Hironishi, Wakayama, 649-63, Japan.

information in the identification of the radical adducts generated during ADP/Fe^{3+}-stimulated microsomal lipid peroxidation.

MATERIALS AND METHODS

Materials

α-(4-Pyridyl-1-oxide)-*N-tert*-butylnitrone (4-POBN) was obtained from Sigma Chem. Co. (St Louis, MO, USA). Sep-pak C_{18} was from Waters Associates (Milford, Massachusetts, USA). Ethylhydrazine oxalate was purchased from Fluka Chem. Co. (Ronkonkoma, New York, USA). Pentylhydrazine oxalate was synthesized according to the method of Gever and Hayes.[23] Millex-GS filters (0.22 μm) were purchased from Millipore Co. (Bedford, MA, USA). Bio-Rad protein assay reagent was from Bio-Rad Laboratories (Richmond, CA, USA). All other chemicals used were commercial products of the highest grade available.

Preparation of Rat-Liver Microsomal Fraction

Male Sprague–Dawley rats, body weight 350–400 g, were used in the experiments. Livers of anesthetized rats (Nembutal) were perfused *in situ* with physiological saline to remove blood prior to homogenization. The livers were suspended in 3 volumes 150 mM KCl, 50 mM Tris buffer (pH 7.4). Homogenization was performed in a Potter tissue grinder with a Teflon pestle. The liver homogenate was centrifuged at 8750 *g* for 15 min. at 5°C. The supernatant fraction was then centrifuged at 165 000 *g* for 38 min. The pellet was resuspended and then centrifuged twice again at 165 000 *g*. The protein concentration of the microsomal suspension was determined using Bio-Rad protein assay reagent.[24]

Reaction

The standard reaction mixture contained 10 ml 0.05 M-sodium phosphate buffer (pH 7.4), 5 ml 0.4 M 4-POBN (in 0.05 M-sodium phosphate buffer, pH 7.4), 1 ml 0.1 M ADP (in 0.05 M-sodium phosphate buffer, pH 7.4), 0.1 ml 10 mM-ferric chloride (in 100 mM HCl), 1 ml 10 mM NADPH (in 0.05 M sodium phosphate buffer, pH 7.4), in 1 ml rat microsomal suspension (0.95 mg/ml protein concentration in sodium phosphate buffer, pH 7.4). Unless otherwise indicated, the reaction was performed for 1 h at 25°C under an air atmosphere. Following the incubation, 8-ml aliquots of the reaction mixture were filtered through a 0.22 μm Millex-GS and applied to a C_{18} Sep-pak. After washing with 10 ml water, the sample was eluted with 1 ml acetonitrile. The eluant was dried under reduced pressure. Each sample was dissolved in 1 ml 20% methanol (v/v) and 10 mM ammonium acetate and injected into HPLC-EPR.

Authentic pentyl- and ethyl-radical adducts of 4-POBN were prepared via the decomposition of pentylhydrazine and ethylhydrazine. The reaction mixtures contained 50 mg 4-POBN, 20 mg pentylhydrazine oxalate (or ethylhydrazine oxalate), and 0.2 mM $CuCl_2$, in 5 ml of 0.05 M carbonate buffer (pH 10.0). After the carbonate buffer was bubbled with nitrogen gas, pentylhydrazine oxalate (or ethylhydrazine oxalate), and $CuCl_2$ were added. The reactions were performed for 2 h at 25°C under an air atmosphere. The reaction mixtures were dried under reduced pressure. After

the samples were dissolved in 4 ml 20% (v/v) methanol and 10 mM ammonium acetate, each 1 ml of the sample was applied to the HPLC-EPR with the semi-preparative μBondapak C_{18} column [300 mm (L) × 10.0 mm (ID)], and the eluant from the EPR active peak was collected. Identification of the ethyl- and pentyl-4-POBN adducts synthesized here was performed using liquid chromatography (LC)/electrospray ionization (ESI)/MS.[25]

HPLC-EPR

HPLC-EPR spectroscopy was performed using an HPLC system equipped with EPR detection as described previously.[21,22] HPLC-EPR was performed using a Waters model 6000A solvent delivery system with a Varian E-104 EPR spectrometer. The EPR spectrometer was connected to the HPLC system by a Teflon tube that passed through the EPR cell. The magnetic field of the EPR spectrometer was fixed at the lowest field peak of the six-line signals detected from 4-POBN radical adducts. The EPR settings were: microwave power, 20 mW; modulation amplitude, 8 G; modulation frequency, 100 kHz; time constant, 1 s. HPLC column conditions were as follows; flow rate, 2.0 ml/min; injection volume, 1.0 ml; gradient elution [solvent A, 10 mM ammonium acetate, 20% methanol (v/v); solvent B, 10 mM ammonium acetate, 80% methanol (v/v)] from 40% B to 100% B in 30 min. The EPR settings were: microwave power, 20 mW; modulation amplitude, 8 G; modulation frequency, 100 kHz; time constant, 1 s; receiver gain, 5×10^4.

RESULTS

EPR analysis was performed on the reaction mixture containing rat liver microsomes, ADP, $FeCl_3$, NADPH and 4-POBN (data not shown). For the complete reaction mixture, typical six-line EPR signals were observed ($a^N = 15.8$ G and $a_\beta^H = 2.6$ G). A similar six-line EPR signal, but of reduced intensity, was also observed in the reaction mixture without microsomes, suggesting the generation of both microsome-dependent and microsome-independent radicals. The microsome-independent radicals may be derived from the ADP or NADPH. No EPR signal was detected for the reaction mixtures without ADP, $FeCl_3$, NADPH or 4-POBN.

In order to investigate what kind of radicals are formed in the reaction mixture, HPLC-EPR analysis was performed. Figure 1-A shows the HPLC-EPR elution pattern of the microsomal incubation with ADP, ferric chloride, NADPH and 4-POBN. Three prominent peaks (1, 2 and 3) were observed at retention times of 13.2 min, 19.6 min, and 24.4 min. The base line change occurring at a retention time of 7 min is due to the sample-injection solvent (1 ml), and the change in solvent composition of the mobile phase caused a further gradual base line drift which started at a retention time of 8 min. Peak 2 was also detected in the reaction mixture without the microsomal fraction, suggesting that peak 2 is not a microsome-derived radical. Peak 2 may correspond to ADP- or NADPH-derived radicals (see above). Peaks 1 and 3 disappeared from the HPLC-EPR elution pattern when the sample was incubated anaerobically (Figure 1-D). In order to determine whether peak 1 and 3 correspond to the ethyl-and pentyl-radical adducts of 4-POBN, the authentic ethyl and pentyl adducts of 4-POBN (Figure 2-B,C) were analysed using HPLC-EPR under the same conditions as the microsomal system. Peaks 1 had the same retention

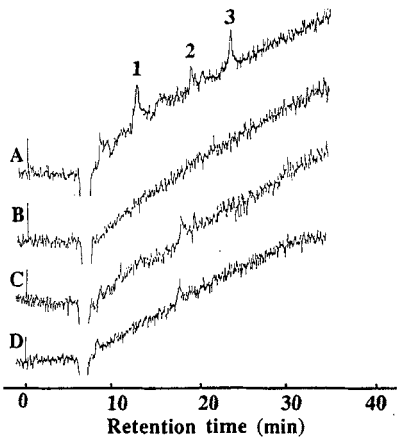

FIGURE 1 HPLC-EPR analysis of the microsomal suspension treated with ADP, NADPH and Fe^{3+}. HPLC-EPR and reaction conditions were as described under Materials and Methods. (A) Complete reaction mixture; (B) without 4-POBN; (C) without microsomal suspension; (D) complete reaction mixture under anaerobic conditions.

time as the 4-POBN ethyl-radical adduct, and Peak 3 showed the same retention time as the pentyl-radical adduct, suggesting that peaks 1 and 3 are the ethyl- and pentyl-radical adducts, respectively.

DISCUSSION

In this experiment we have detected the ethyl and pentyl radical adducts of 4-POBN by HPLC-EPR during lipid peroxidation of rat liver micromoses by the ADP, NADPH and ferric chloride system. The pentyl and ethyl radicals can be formed following the

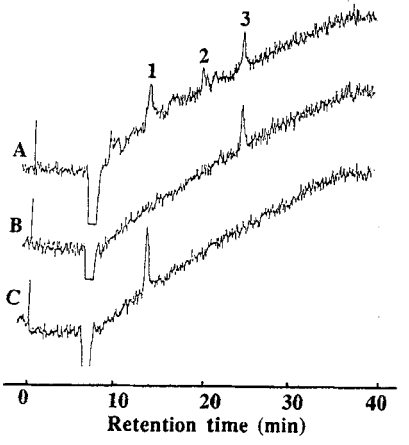

FIGURE 2 HPLC-EPR elution pattern of complete reaction mixture, authentic ethyl radical, and authentic pentyl radical adduct. Reaction and HPLC-EPR conditions were as described in Materials and Methods. (A) Complete reaction mixture; (B) authentic ethyl radical; (C) authentic pentyl radical.

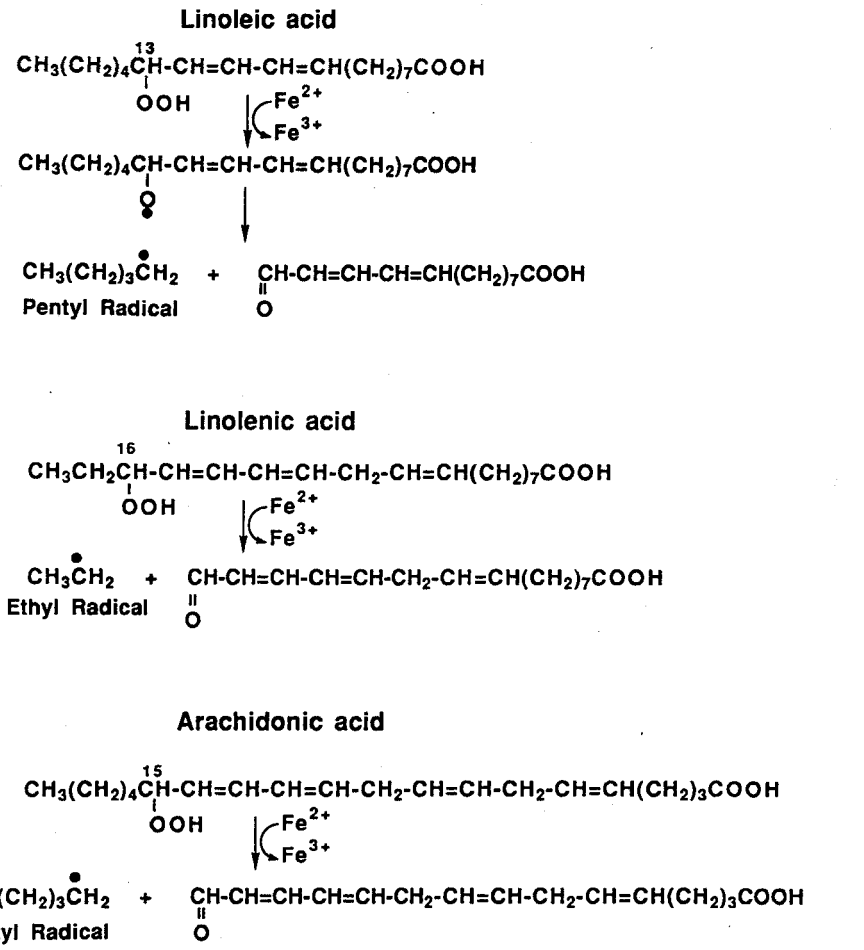

FIGURE 3 A possible mechanism for the formation of ethyl radicals and pentyl radicals from linoleic acid, linolenic acid, and arachidonic acid.

schemes shown in Figure 3. When linoleic acid, linolenic acid and arachidonic acid are peroxidized at C-13 for linoleic acid, at C-16 for linolenic acid, and at C-15 for arachidonic acid (Figure 3), β-scission of the corresponding hydroperoxides is proposed to occur through the alkoxyl radical intermediate. This produces the pentyl radical from linoleic acid and arachidonic acid, and ethyl radical from linolenic acid.[26] By hydrogen abstraction, these radicals would form ethane and pentane, which have long been measured as indicators of lipid peroxidation.[27,28] Presumably, lipid-derived radicals such as L·, LO·, and LOO· are formed in this reaction mixture because ethane and pentane are formed *via* these free radicals.[29] Either a lower rate of trapping or a short life time for the respective radical adducts, however, results in failure to detect these radical adducts under our conditions. Indeed, Janzen *et al.*, reported the α-phenyl-*N*-*tert*-butylnitrone (PBN) radical adduct with LO· in microsomal suspension treated by CCl_4 by using deuterated PBN[7] in addition to the primary alkyl

fragment radicals. The primary alkyl fragment radicals may correspond to ethyl and pentyl radicals in our report. In a similar manner, it is possible to form pentenyl radical from 13-hydroperoxy-linolenic acid.[30] However, a peak for 4-POBN-pentenyl radical adduct was not detected in this reaction mixture. If the 4-POBN-pentenyl radical adduct, which is obtained from reaction of lipoxygenase with linolenic acid,[30] were present, it would have eluted between peak 2 and peak 3.

EPR spin trapping technique is a powerful technique to detect free radicals in biological and chemical systems.[31,32] In general, identification of the radical adducts has been based on their hyperfine coupling constants. However, identical hyperfine coupling constants, which are very solvent-dependent, have been observed for different radical adducts. This confounds the identification of radical adducts in biological systems. HPLC-EPR retention times are additional useful information for the identification of the radical adducts. Although mass spectrometry could in principle give unambiguous structural identification of these radical adducts,[33,34] the concentrations of the radical adducts formed in this system were too low to obtain mass spectra.

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