J. Biochem. **139**, 671–676 (2006)
doi:10.1093/jb/mvj082

Enhancement by Cigarette Smoke Extract of the Radical Formation in a Reaction Mixture of 13-Hydroperoxide Octadecadienoic Acid and Ferric Ions

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Received January 12, 2006; accepted February 2, 2006

The effects of cigarette smoke extract on radical formation were examined in reaction mixtures containing 13-hydroperoxide octadecadienoic acid (13-HPODE), $FeCl_3$, cigarette smoke extract, ethylenediaminetetraacetic acid (EDTA), α-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone (4-POBN), and phosphate buffer (pH 7.4). Cigarette smoke extract enhanced the formation of both 7-carboxyheptyl and pentyl radicals in the reaction. Ferric ions were reduced in the reaction mixture, suggesting that cigarette smoke extract enhances the formation of 7-carboxyheptyl and pentyl radicals by reducing ferric irons. Although there is a large body of evidence supporting the involvement of radicals such as the semiquinone radical, hydroxyl radical, superoxide radical, nitric oxide radicals in smoking-related diseases, the enhancement by cigarette smoke of lipid-derived radical formation, which we first report here, may be one of the other causes of smoking-related diseases.

Key words: fatty acid, lipid, mass spectrometry, HPLC, ESR, radicals.

Cigarette smoke may be a major cause of human lung cancer and other respiratory diseases (*1*). Although the hazards of cigarette smoke seem to be conclusive (*2*, *3*), the mechanisms of cigarette smoke–induced chemical reactions are not yet well understood.

Hydrogen peroxide (*4*, *5*), semiquinone radical (*6*), hydroxyl radical (*7*), superoxide radical (*8*, *9*), and nitric oxide radicals (*10*) have been observed in cigarette tar and smoke, and, there is a large body of evidence supporting the involvement of these radicals in smoking-related diseases (*11–16*). Although cigarette smoke contains many radical species, there are few reports examining whether or not cigarette smoke extract enhances the formation of lipid-derived radicals.

Linoleic acid hydroperoxide and hydroperoxides of other unsaturated fatty acids are extremely toxic when injected into mice (*17*). Subcutaneous or intravenous injection of linoleic acid peroxide results in marked lesions in the intima of the aorta (*18*, *19*). Lipid peroxide–derived radicals seem to be related to the toxicity of lipid peroxides. Indeed, lipid-derived free radicals, which form in the reaction of lipid peroxides with transition metals, are known to cause damage to biomembranes, proteins and the other biomolecules (*20–22*).

In this report, the effects of cigarette smoke extract on lipid-derived radical formation are examined in reaction mixtures containing 13-hydroperoxide octadecadienoic acid and ferric ions using electron spin resonance (EPR), high performance liquid chromatography–electron spin resonance (HPLC-EPR), and high performance liquid chromatography–electron spin resonance-mass spectrometry (HPLC-EPR-MS) combined use of the spin trapping technique. HPLC-EPR-MS involves the sequential coupling of HPLC, EPR, and MS so that separation, radical adduct detection, and mass spectrometric determination occur on line.

EXPERIMENTAL AND METHODS

Materials—Linoleic acid (9,12-octadecadienoic acid), superoxide dismutase [EC 1.15.1.1] (SOD), citric acid, and soybean lipoxygenase [EC 1.13.11.12] type V were obtained from Sigma Chemical Co. (St. Louis, MO, USA). α-(4-Pyridyl-1-oxide)-*N-tert*-butylnitrone (4-POBN) was purchased from Tokyo Kasei Kogyo, Ltd. (Tokyo, Japan). Ethylenediaminetetraacetic acid disodium salt (EDTA) and ferric chloride were obtained from Wako Chemicals Inc. (Osaka, Japan). 1,10-Phenanthroline and diethylenetriaminepentaacetic acid (DETAPAC) were purchased from Nakarai Chemicals, Ltd. (Kyoto, Japan). Adenine 5′-diphosphate disodium salt (ADP) was obtained from Oriental Yeast Co. Ltd. (Tokyo, Japan). 13-Hydroperoxide octadecadienoic acid (13-HPODE) was prepared according to the previously outlined procedure (*23*).

Cigarette Smoke Extract—Commercial cigarettes were store in sealed packages at room temperature and opened before use. Five cigarettes were smoked at a continuous flow rate of 1,000 ml/min (Fig. 1), the smoke components were collected on gossypium absorbens. The smoke components were then extracted with 5 ml of 50 mM phosphate buffer (pH 7.4) from the gossypium absorbens.

Complete Reaction Mixture of 13-HPODE with Ferric Ions—The complete reaction mixture contained cigarette smoke extract (8% of the total volume of the reaction mixture), 0.08 mM $FeCl_3$, 0.14 mM 13-HPODE, 0.08 mM EDTA, 0.1 M 4-POBN, and 28 mM phosphate buffer

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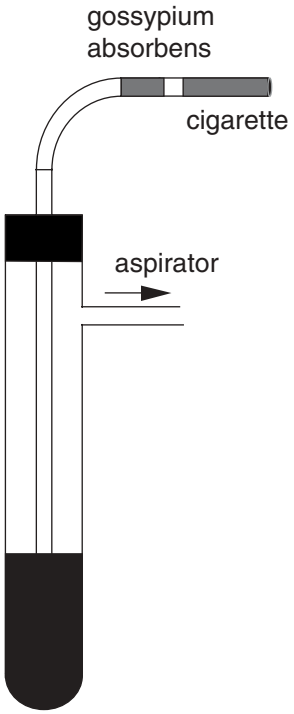


Fig. 1. **Apparatus for collecting smoke extract.**

(pH 7.4). The reaction was started by adding $FeCl_3$, and continued for 30 min at 25°C. For EPR measurement, 0.25 ml of the reaction mixture was analyzed. After filtration through a MILLEX-GS (MILLIPORE, Bedford, MA, USA), 2.5 ml of the reaction mixture was applied to HPLC-EPR and HPLC-EPR-MS.

EPR, HPLC-EPR, and HPLC-EPR-MS Analyses—EPR, HPLC-EPR, and HPLC-EPR-MS analyses were performed according to the previously outlined procedures (*24*) except for the mobile phase. For HPLC-EPR and HPLC-ESR-MS, two solvents were used: solvent A, 50 mM acetic acid (solvent A) and 50 mM acetic acid/acetonitrile (20:80, v/v; solvent B). A combination of isocratic and linear gradients was used: 0–40 min, 100% A to 0% A (linear gradient) at a flow rate 2.0 ml/min; 40–60 min, 100% B (isocratic) at a flow rate 2.0 ml/min.

Reduction of Ferric Ions by Cigarette Smoke Extract—The complete reaction mixture contained in a total volume of 2.0 ml, 0.16 ml of cigarette smoke extract (in 50 mM phosphate buffer), 0.16 ml of 1 mM $FeCl_3$ (in 10 mM HCl), 0.16 ml of 1 mM EDTA (in H_2O), 0.01 ml of 1 M 1,10-phenanthroline (in ethanol), and 1.51 ml of 50 mM phosphate buffer (pH 7.4). The reaction was started by adding $FeCl_3$. After 30 min at 25°C, the visible spectra of the reaction mixtures were measured. All spectrophotometric measurements were obtained using cuvettes with a 10 mm light path, in a Shimadzu UV-160A UV-VISIBLE RECORDING SPECTROPHOTOMETER at 25°C.

RESULTS AND DISCUSSION

Effects of Cigarette Smoke Extract on Radical Formation in the Reaction of 13-HPODE with Ferric Ions—The EPR spectra of the complete reaction mixture of 13-HPODE with ferric ions were measured (Fig. 2). A prominent EPR peak (a^N = 1.58 mT and $a^H\beta$ = 0.26 mT) was observed in the complete reaction mixture (Fig. 2A). In the absence of the cigarette smoke extract, the EPR signal decreased to 10.5 ± 1.0% of that in the complete reaction mixture (mean of three experiments ± SD) (Fig. 2B), suggesting that cigarette smoke extract enhances the formation of

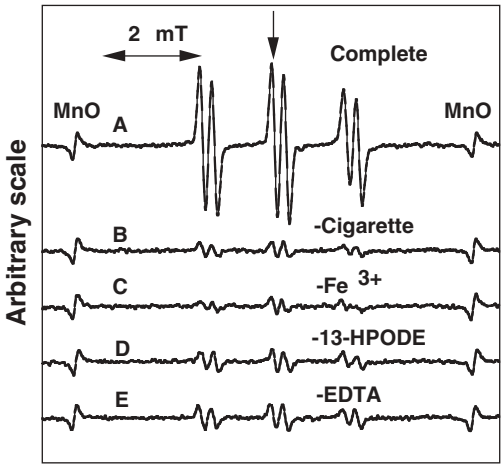


Fig. 2. **EPR spectra of the reaction mixtures of 13-HPODE with the smoke extract.** EPR and reaction conditions were as described in "MATERIALS AND METHODS." A, complete reaction mixture; B, complete reaction mixture without cigarette smoke extract; C, complete reaction mixture without ferric ions; D, complete reaction mixture without 13-HPODE; E, complete reaction mixture without EDTA.

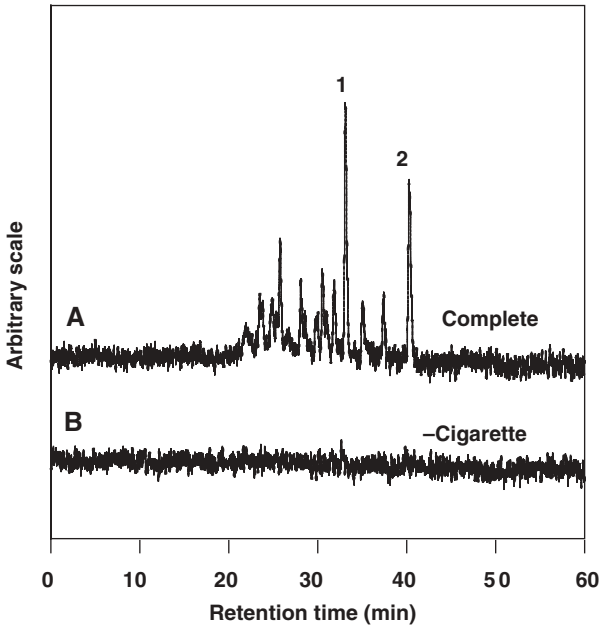


Fig. 3. **HPLC-EPR analyses of reaction mixtures of 13-HPODE and ferric ions in the presence of cigarette smoke extract.** HPLC-EPR and reaction conditions were as described in "MATERIALS AND METHODS." A, complete reaction mixture; B, complete reaction mixture without cigarette smoke extract.

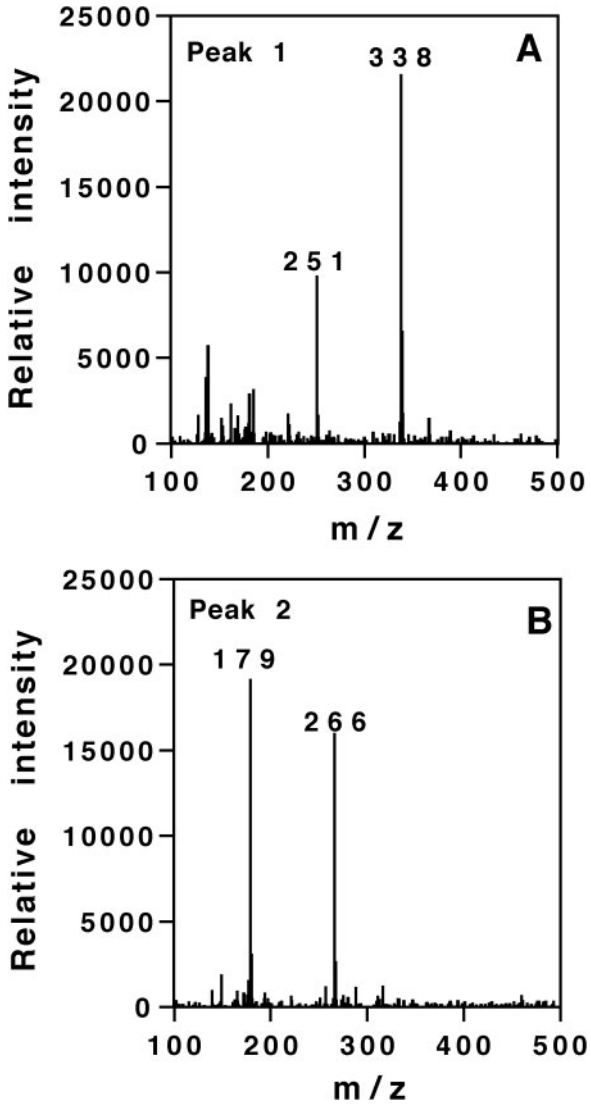


Fig. 4. **HPLC-EPR-MS analyses of the reaction mixtures of 13-HPODE and ferric ions in the presence of cigarette smoke extract.** HPLC-EPR-MS and reaction conditions were as described in "MATERIALS AND METHODS." A, mass spectrum of peak 1; B, mass spectrum of peak 2.

13-HPODE–derived radicals. In the absence of ferric ion (Fig. 2C) [or 13-HPODE (Fig. 2D), or EDTA (Fig. 2E)], the EPR signal decreased to 9.9 ± 1.3% (or 14.1 ± 1.4%, or 16.2 ± 0.9%) of that of in the complete reaction mixture (mean of three experiments ± SD).

HPLC-EPR and HPLC-EPR-MS Analyses of the Complete Reaction Mixture—In order to know the kinds of radicals formed in complete reaction mixture, HPLC-EPR and HPLC-EPR-MS analyses were performed. Two prominent peaks were observed with the retention times of 33.1 min (peak 1) and 40.4 min (peak 2) in the HPLC-EPR elution profile (Fig. 3A). These peaks were hardly detected in the elution profile of the complete reaction mixture without cigarette smoke extract (Fig. 3B).

HPLC-EPR-MS analysis of peak 1 gave ions at *m/z* 251 and *m/z* 338 (Fig. 4A). The ion *m/z* 338 corresponds to the protonated molecular ion of the 4-POBN/7-carboxyheptyl radical adduct, $(M+H)^+$. The fragment ion at

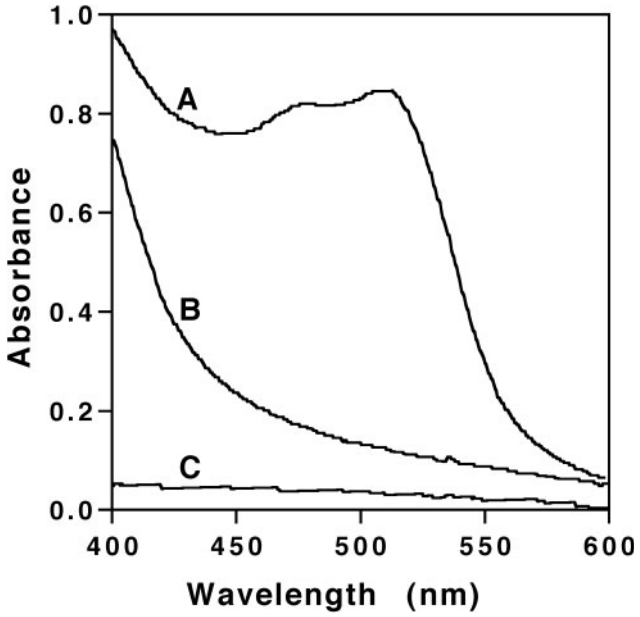


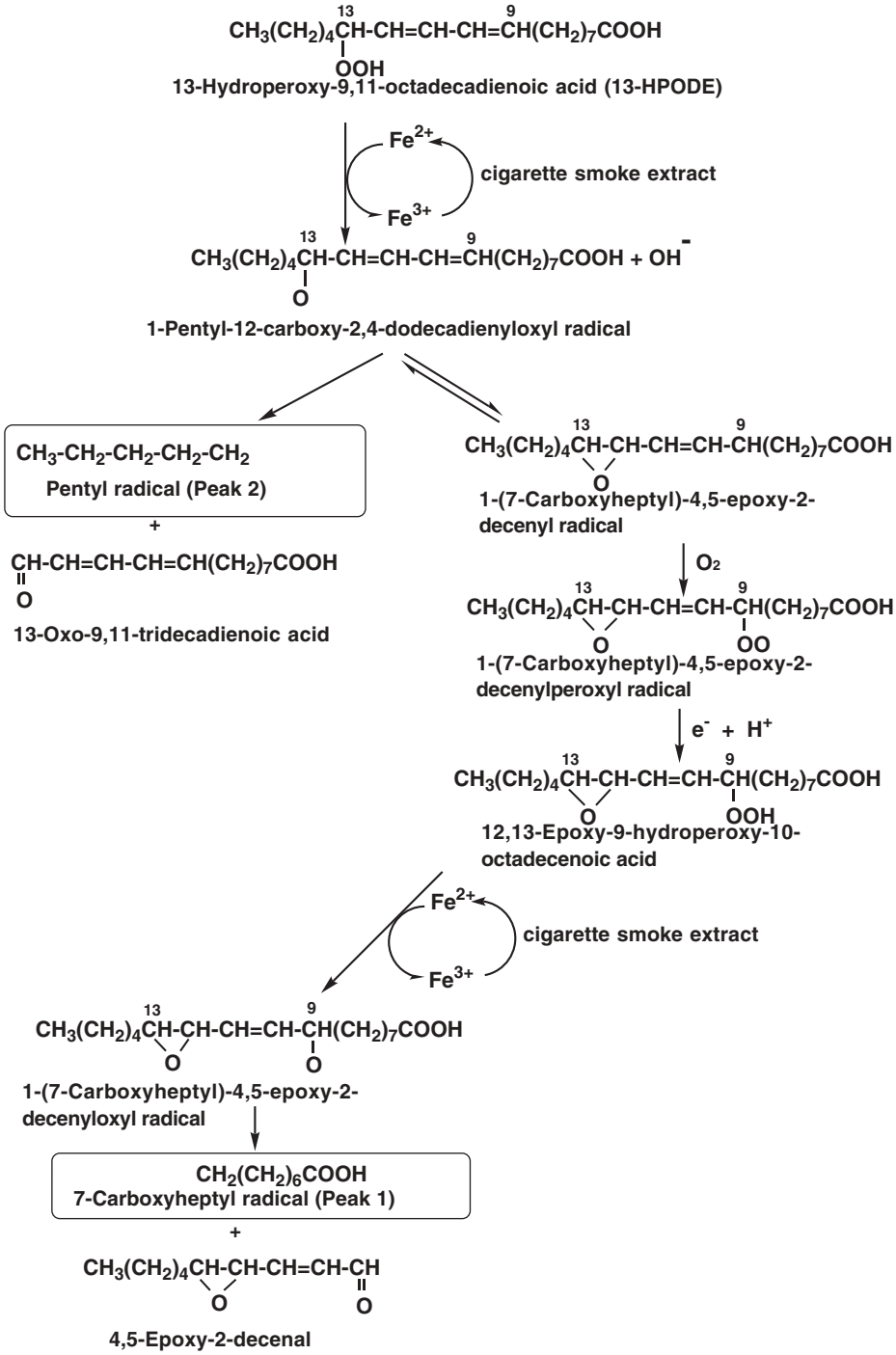
Fig. 5. **Visible spectra of reaction mixtures containing $FeCl_3$, EDTA, cigarette smoke extract, and 1,10-phenanthroline.** Visible absorbance measurements and reaction conditions were as described in "MATERIALS AND METHODS." A, complete reaction mixture; B, complete reaction mixture without 1,10-phenanthroline; C, complete reaction mixture without cigarette smoke extract.

m/z 251 corresponds to the loss of $[(CH_3)_3C(O)N]$ from the protonated molecular ion. The assignment was confirmed by comparing the retention time with that of the authentic 4-POBN/7-carboxyheptyl radical adduct. The authentic compound was synthesized in a reaction of linoleic acid with soybean lipoxygenase and identified by MS/MS analysis (*24*).

HPLC-EPR-MS analysis of peak 2 gave ions at *m/z* 179 and *m/z* 266 (Fig. 4B). The ion *m/z* 266 corresponds to the protonated molecular ions of 4-POBN/pentyl radical adducts, $(M+H)^+$. The fragment ion at *m/z* 179 corresponds to the loss of $[(CH_3)_3C(O)N]$ from the protonated molecular ion. The assignment was confirmed by comparing the retention time with that of the authentic 4-POBN/pentyl radical adduct. The authentic compound was synthesized in a reaction of pentylhydrazine with Cu^{2+} (*25*).

A possible reaction path for the formation of 7-carboxyheptyl and pentyl radicals is shown in Scheme 1. Previous studies have shown the formation of 7-carboxyheptyl and pentyl radicals in reaction mixtures of 13-HPODE and ferrous ions (*26–28*). Ferrous ions participate in the reaction between 13-hydroperoxy-9,11-octadecadienoic acid (13-HPODE) and 1-pentyl-12-carboxy-2,4-dodecadienyloxyl radicals [or between 12,13-epoxy-9-hydroperoxy-10-octadecenoic acid and 1-(7-carboxyheptyl)-4,5-epoxy-2-decenyloxyl radicals] (Scheme 1).

Reduction of Ferric Ions by Cigarette Smoke Extract—To investigate the supply of ferrous ions, the oxidative state of the iron ions was examined in the reaction mixture containing cigarette smoke extract, $FeCl_3$, EDTA, 1,10-phenanthroline using a UV-visible spectrophotometer. The reaction mixture gave a visible spectrum with λ_{max} of 512 nm, while no peak at 512 nm was present



Scheme 1. **A possible mechanism for the enhancement of the formation of 13-HPODE–derived radicals by cigarette smoke extract.**

in the reaction mixture without cigarette smoke extract (Fig. 5). The results indicate that ferric ions are reduced by cigarette smoke extract, because the λ_{max} of 512 nm is due to the Fe(II)-1,10-phenanthroline complex (*29*), suggesting that the cigarette smoke extract may enhance the reaction by reducing ferric irons.

Superoxide anions were previously detected in cigarette smoke extracts (*8*, *9*). Superoxide anions possibly reduce ferric ions (Eq. 1).

$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2 \quad (1)$$

Zang *et al*. reported that reductants such as hydroquinone and semiquinone are contained in cigarette smoke extracts (*30*). These species also may reduce ferric ions and enhance the formation of 13-HPODE–derived radicals.

Effects of SOD on the Formation of the 13-HPODE–Derived Radicals in the Complete Reaction Mixture—In order to know whether or not superoxide anions participate in the enhancement of 13-HPODE–derived radical formation by cigarette smoke extract, SOD was added to the complete reaction mixture. The SOD partially inhibited the formation of

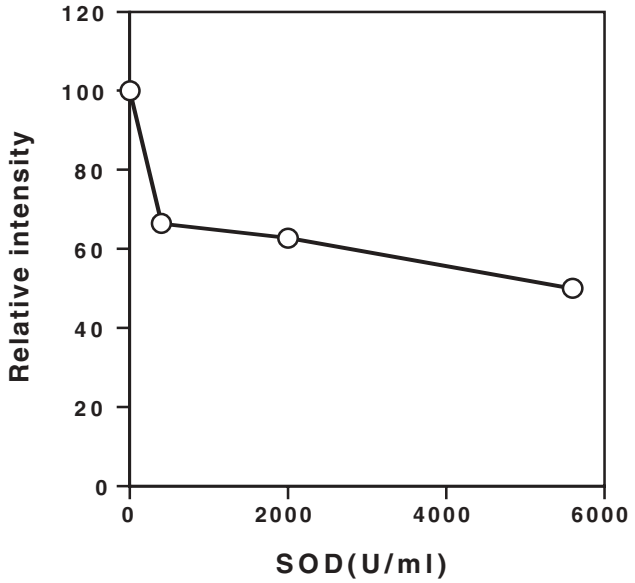


Fig. 6. **Effects of SOD on the formation of 13-HPODE-derived radicals.** EPR and reaction conditions were as described in "MATERIALS AND METHODS" except for the addition of SOD. The reaction mixtures contained 0 U/ml, 400 U/ml, 2,000 U/ml, or 5,600 U/ml of SOD.

13-HPODE–derived radicals (Fig. 6), suggesting that the superoxide anions participate in the enhancement.

Effects of Some Chelators on the Formation of 13-HPODE–Derived Radicals in the Complete Reaction Mixture—Since in the absence of the EDTA, the EPR peak height decreased to 16.2 ± 0.9% of that of the complete reaction mixture (mean of three experiments ± SD) (Fig. 2E), EDTA seems to be essential for the enhancement of the formation of 13-HPODE–derived radicals. In order to examine the effects of some endogenous chelators on the formation of 13-HPODE–derived radicals, ADP (or citrate, or DETAPAC) was added to the complete reaction mixture instead of EDTA. The addition of ADP (or citrate) resulted in an increase in the EPR peak height [162 ± 10% (or 158 ± 10%) of the complete reaction mixture without EDTA] (mean of three experiments ± SD). ADP and citrate may participate in the enhancement of 13-HPODE–derived radical formation *in vivo* by cigarette smoke extract. On the other hand, DETAPAC decreased the EPR peak height to 62 ± 4% of the complete reaction mixture without EDTA (mean of three experiments ± SD).

REFERENCES

1. Auerobach, O., Hammond, E.C., Garfinkel, L., and Benante, C. (1972) Relation of smoking and age to emphysema. Whole-lung section study. *N. Eng. J. Med.* **286**, 853–857
2. Bengali, Z.H. (1987) NHLBI workshop summary. Effects of tobaoco smoke components on cellular and biochemical processes in the lung. *Am. Rev, Respir. Dis.* **136**, 1058–1064
3. Wynder, E.L. (1988) Tobacco and health: a review of the history and suggestions for public health policy. *Public Health Reports* **103**, 8–18
4. Nakayama, T., Kodama, M., and Nagata, C. (1984) Generation of hydrogen peroxide and superoxide anion radical from cigarette smoke. *Gann* **75**, 95–98
5. Nakayama, T., Church, D.F., and Pryor, W.A. (1989) Quantitative analysis of the hydrogen peroxide formed in aqueous cigarette tar extracts. *Free Radic. Biol. Med.* **7**, 9–15
6. Pryor, W.A., Hales, B.J., Premovic, P. l., Church, D.F. (1983) The radicals in cigarette tar: Their nature and suggested physiological implications. *Science* **220**, 425–427
7. Cosgrove, J.P., Borish, E.T., Church, D.F., and Pryor, W.A. (1985) The metal-mediated formation of hydroxyl radical by aqueous extracts of cigarette tar. *Biochem. Biophys. Res. Commun.* **132**, 390–396
8. Tsuchiya, M., Thompson, D.F.T., Suzuki, Y.J., Cross, C.E., and Packer, L. (1992) Superoxide formed from cigarette smoke impairs polymorphonuclear leukocyte active oxygen generation activity. *Arch. Biochem. Biophys.* **299**, 30–37
9. Tanigawa, T., Yoshikawa, T., Takahashi, S., Naito, Y., and Kondo, M. (1994) Spin trapping of superoxide in aqueous solutions of fresh and aged cigarette smoke. *Free Radic. Biol. Med.* **17**, 361–365
10. Shinagawa, K., Tokimoto, T., and Shirane, K. (1998) Spin trapping of nitric oxide in aqueous solutions of cigarette smoke. *Biochem. Biophys. Res. Commun.* **253**, 99–103
11. Carp, H., Miller, F., Hoidal, J.R., and Janoff, A. (1982) Potential mechanism of emphysema: α_1-Proteinase inhibitor recovered from lungs of cigarette smokers contains oxidized methionine and has decreased elastase inhibitory capacity. *Proc. Natl. Acad. Sci. USA* **79**, 2041–2045
12. Nakayama, T., Kaneko, M., Kodama, M., and Nagata, C. (1985) Cigarette smoke induces DNA single-strand breaks in human cells. *Nature* **314**, 462–464
13. Church, D.F., and Pryor, W.A. (1985) Free-radical chemistry of cigarette smoke and its toxicological implications. *Environ. Health Perspect.* **64**, 111–126
14. Frei, B., Forte, T.M., Ames, B.N., and Cross, C.E. (1991) Gas phase oxidants of cigarette smoke induce lipid peroxidation and changes in lipoprotein properties in human blood plasma. *Biochem. J.* **277**, 133–138
15. Kirkham, P.A., Spooner, G., Ffoulkes-Jones, C., and Calvez, R. (2003) Cigarette smoke triggers macrophage adhesion and activation: Role of lipid peroxidation products and scavenger receptor. *Free Radic. Biol. Med.* **35**, 697–710
16. Panayiotidis, M.I., Stabler, S.P., Allen, R.H., Ahmad, A., and White, C.W. (2004) Cigarette smoke extract increases S-adenosylmethionine and cystathionine in human lung epithelial-like (A549) cells. *Chem-Biol. Interactions* **147**, 87–97
17. Horgan, V.J., Philpot, J. St L., Porter, B.W. and Roodyn, D.B. (1957) Toxicity of autooxidized squalene and linoleic acid, and of simpler peroxides, in relation to toxicity of radiation. *Biochem. J.* **67**, 551–558
18. Cutler, M.G. and Schneider, R. (1974) Linoleate oxidation products and cardiovascular lesions. *Atherosclerosis* **20**, 383–394
19. Yagi, K., Ohkawa, H., Ohishi, N., Yamashita, M. and Nakashima, T. (1981) Lesion of aortic intima caused by intravenous administration of linoieic acid hydroperoxide. *J. Appl. Biochem.* **3**, 58–65
20. Gardner, H.W. (1975) Decomposition of linoleic acid hydroperoxides. Enzymic reactions compared with nonenzymic. *J. Agr. Food Chem.* **23**, 129–136
21. Marnett, L.J. (1987) Peroxyl free radicals: potential mediators of tumor initiation and promotion. *Carcinogenesis* **8**, 1365–1373
22. O'Brien, P.J. (1988) in *Cellu]ar Antioxidant Defense Mechanism*. Vol. 1 (Chow, C.K., ed.) pp. 73–87, CRCPress, Boca Raton
23. Teng, J.I. and Smith, L.L. (1985) High-performance liquid chromatography of linoleic acid hydroperoxides and their corresponding alcohol derivatives. *J. Chromatogr.* **350**, 445–451

24. Iwahashi, H., Deterding, L.J., Parker, C.E., Mason, R.P. and Tomer, K.B. (1996) Identification of radical adducts formed in the reactions of unsaturated fatty acids with soybean lipoxygenase using continuous flow fast atom bombardment with tandem mass spectrometry. *Free Radic. Res.* **25**, 255–274
25. Iwahashi, H., Albro, P.W., McGown, S.R., Tomer, K.B. and Mason, R.P. (1991) Isolation and identification of α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone radical adducts formed by the decomposition of the hydroperoxides of linoleic acid, linolenic acid, and arachidonic acid by soybean lipoxygenase. *Arch. Biochem. Biophys.* **285**, 172–180
26. Iwahashi, H. (2000) Some polyphenols inhibit the formation of pentyl radical and octanoic acid radical in the reaction mixture of linoleic acid hydroperoxide with ferrous ions. *Biochem. J.* **346**, 265–273
27. Iwahashi, H., Nishizaki, K., and Takagi, I. (2002) Cytochrome c catalyses the formation of pentyl radical and octanoic acid radical from linoleic acid hydroperoxide. *Biochem. J.* **361**, 57–66
28. Iwahashi, H., Kumamoto, K., Hirai, T. (2003) The formation of the 7-carboxyheptyl radical from 13-hydroperoxy-9,11-octadecadienoic acid catalyzed by hemoglobin and myoglobin under anaerobic conditions. *J. Biochem.* **133**, 679–685
29. Harvey, A.E., Jr., Smart, J.A., and Amis, E.S. (1955) Simultaneous spectrophotometric determination of iron (II) and total iron with 1,10-phenanthroline. *Anal. Chem.* **27**, 26–29
30. Zang, L.Y., Stone, K., and Pryor, W.A. (1995) Detection of free radicals in aqueous extracts of cigarette tar by electron spin resonance. *Free Radic. Biol. Med.* **19**, 161–167