ELSEVIER

Biophysical Chemistry 77 (1999) 153–160

Biophysical Chemistry

Antioxidant properties of EPC-K1: a study on mechanisms

Taotao Wei[a], Chang Chen[a], Fengmei Li[b], Baolu Zhao[a], Jingwu Hou[a], Wenjuan Xin[a,*], Akitane Mori[c]

[a] *Institute of Biophysics, Academia Sinica, 15 Datun Road, Chaoyang District, Beijing 100101, China*
[b] *Institute of Low Energy Nuclear Physics, Beijing Normal University, Beijing, China*
[c] *Department of Neuroscience, Institute of Molecular and Cellular Medicine, Okayama University Medical School, Okayama, Japan*

Received 13 July 1998; received in revised form 5 February 1999; accepted 5 February 1999

Abstract

Scavenging effects of L-ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethytridecyl)-2*H*-1-benzopyran-6-yl-hydrogen phosphate] potassium salt (EPC-K1) on hydroxyl radicals, alkyl radicals and lipid radicals were studied with ESR spin trapping techniques. The inhibition effects of EPC-K1 on lipid peroxidation were assessed by TBA assay. The kinetics of EPC-K1 reacting with hydroxyl radicals and linoleic acid radicals were studied by pulse radiolysis. The active site of EPC-K1 and the structure-antioxidative activity relationships were discussed. The superoxide radicals scavenging capacity of the brain homogenate of EPC-K1-treated rats was measured. The results revealed that in comparison with Trolox and vitamin C, EPC-K1 showed better overall antioxidative capacity in vitro and in vivo. EPC-K1 was a moderate scavenger on hydroxyl radicals and alkyl radicals, a potent scavenger on lipid radicals, and an effective inhibitor on lipid peroxidation. EPC-K1 could react with hydroxyl radicals with a rate constant of 7.1×10^8 dm^3 mol^{-1} s^{-1} and react with linoleic acid radicals with a rate constant of 2.8×10^6 dm^3 mol^{-1} s^{-1}. The active site of EPC-K1 was the enolic hydroxyl group. After administration of EPC-K1, the ability of rat brain to scavenge superoxide radicals was significantly increased. The potent scavenging effects of EPC-K1 on both hydrophilic and hydrophobic radicals were relevant with its molecular structure, which consisted of both hydrophilic and hydrophobic groups.

Keywords: EPC-K1; Antioxidant; ESR; Spin trapping; Pulse radiolysis; Reactive oxygen species; Lipid peroxidation

* Corresponding author. Tel.: +86-10-64888576; fax: +86-10-64877837; e-mail: xinzhsc@sun5.ibp.ac.cn

0301-4622/99/$ - see front matter
PII: S0301-4622(99)00017-4

1. Introduction

It has been well accepted that reactive oxygen species (ROS) are associated with many diseases [1]. The central nervous system (CNS) is especially prone to oxidative damage for several reasons. First, the brain has a very high oxygen consumption ratio; second, neuronal cell membrane is rich in polyunsaturated fatty acid side chains; third, the antioxidant defense systems in the CNS are relatively poor. During recent years, the role of ROS in the pathogenesis of neurodegenerative disorders, such as Alzheimer's disease, amyotrophic lateral sclerosis and Parkinson's disease has attracted more and more attention [2–4], and antioxidants may be effective drugs for these diseases [5,6]. L-Ascorbic acid (vitamin C) and α-tocopherol (vitamin E), two endogenous antioxidants, are important members of non-enzymatic antioxidant defense systems. However, vitamin E is insoluble in water, several water-soluble analogs and derivatives of it are synthesized in order to improve its absorption. EPC-K1 is a novel phosphate ester derivative of vitamin C and vitamin E, which is soluble in both water and lipid. It has been reported that EPC-K1 can act as an inhibitor on lipid peroxidation [7] and can protect the brain from ischemia-reperfusion injury [8,9], but the antioxidant mechanisms are still not clear. In this study, the antioxidant capacity of EPC-K1 in vitro and in vivo was assessed and the kinetics of EPC-K1 reacting with hydroxyl radicals and linoleic acid radicals were studied. The antioxidant mechanisms were also discussed.

2. Materials and methods

2.1. Materials

Male Wistar rats weighing approximately 250 g were purchased from Beijing Medical University. Hypoxanthine, xanthine oxidase and spin traps 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) were purchased from Sigma; 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Aldrich; 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) were purchased from Wako Pure Chemical Industries Ltd. Linoleic acid was purchased from Fluka. L-Ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethytridecyl)-2*H*-1-benzopyran-6-yl-hydrogen phosphate] potassium salt (EPC-K1) was a generous gift from Senju Pharmaceutical Co. Ltd., Japan. Other reagents made in China were of analytical grade.

2.2. Scavenging effect of EPC-K1 on hydroxyl radicals

Hydroxyl radicals were generated by photolysis of hydrogen peroxide and trapped by DMPO [10]. Reaction mixtures with a total volume of 50 μl containing 0.08 mol/l DMPO, 0.01 mol/l H_2O_2 and antioxidants were irradiated with a UV lamp (wavelength: 254 nm; power: 200 W; the distance between the light source and the sample: 1.2 m) for 1 min and the ESR spectra were recorded immediately.

2.3. Scavenging effect of EPC-K1 on alkyl radicals

Alkyl radicals were generated by the decomposition of AAPH and trapped by POBN. Reaction mixtures with a total volume of 50 μl containing 0.03 mol/l POBN, 5 mmol/l AAPH and antioxidants were incubated at 37°C for 30 min and the ESR spectra were recorded immediately. In order to exclude the involvement of molecular oxygen, all solutions were prepared with N_2-saturated PBS (pH 7.4).

2.4. Scavenging effect of EPC-K1 on lipid radicals

Lipid radicals were generated by Fe^{3+}/Fe^{2+}-initiated lipid peroxidation in intact cerebellar neurons and trapped by POBN [11]. Rat cerebellar neurons were prepared as described [12] and resuspended in air-saturated PBS (pH 7.4). Aliquots of cell suspension containing 2×10^6 cells were incubated with EPC-K1, Trolox or vitamin C at 37°C for 10 min and then 0.03 mol/l POBN, 0.5 mmol/l $FeSO_4$ and 1.5 mmol/l $FeCl_3$ were added. After incubation at 37°C for 30 min, the ESR spectra were recorded immediately.

2.5. Scavenging effect of rat brain homogenate on superoxide radicals

The effect of administration of EPC-K1 in vivo on the antioxidant capacity of rat brain was assessed by measuring the superoxide radical scavenging activity using ESR spin trapping techniques as previous reports [13,14].

Male Wistar rats weighing approximatley 250 g were used in the in vivo experiment. EPC-K1, Trolox and vitamin C were administrated i.p. at doses of 20 mg/kg everyday for a week. On the eighth day, the rats were killed by decapitation and the brain were dissected out, rinsed with ice-cold phosphate-buffered saline (PBS), minced, and homogenized with PBS. After centrifugation at 2000 $\times$ g for 10 min, the supernatant was used as brain homogenate. The protein concentration was determined by biuret method using bovine serum albumin as standard. The protein concentration of all samples were adjusted to 20 mg/ml with PBS.

Superoxide radicals were generated with hypoxanthine and xanthine oxidase. Reaction mixtures containing 0.5 mmol/l hypoxanthine, 0.96 mmol/l DETAPAC, 5 mg/ml brain homogenate, 0.1 mol/l DMPO and 0.05 U/ml xanthine oxidase were mixed and transferred into quartz capillaries for ESR measurement. The ESR spectra were recorded 40 s after the addition of xanthine oxidase.

2.6. ESR measurement conditions

All ESR spectra were recorded at room temperature (298 K) by a Varian E-109 spectrometer with measurement conditions as: X-band, central magnetic field 325 mT, sweep width 20 mT, microwave power 20 mW, 100 kHz modulation with amplitude 0.1 mT (DMPO as spin trap) or 0.2 mT (POBN as spin trap), time constant 0.128 s.

2.7. Inhibition effect of EPC-K1 on peroxyl-initiated lipid peroxidation

Brain mitochondria were prepared as described [15] from Wistar rats weighing approximately 250 g and resuspended in air-saturated PBS (pH 7.4). After freezing-thawing for three cycles and sonication at 0°C for 1 min, the submitochondrial particles obtained were used in the experiments. The protein concentration was determined by the biuret method using bovine serum albumin as standard.

Peroxyl radicals, which act as lipid peroxidation initiators, were generated by the decomposition of AAPH (water-soluble initiator) or AMVN (lipid-soluble initiator). For AAPH-initiated lipid peroxidation, aliquots of submitochondrial particles containing 0.8 mg protein were incubated with EPC-K1, Trolox or vitamin C at 37°C for 10 min and then AAPH (5 mmol/l) was added. After incubation at 37°C for 30 min, the TBARS were measured by TBA assay [10]. For AMVN-initiated lipid peroxidation, AMVN dissolved in chloroform was transferred into a glass homogenizer, and the solvent was removed by nitrogen flux to form a thin layer of AMVN on the wall. Suspensions of submitochondrial particles were added into the homogenizer and the mixtures were mechanically homogenized and then sonicated at 0°C for 30 s. Aliquots of submitochondrial particles containing 1 mg protein and 5 mmol/l AMVN were incubated with EPC-K1, Trolox or vitamin C at 0°C for 20 min and then were incubated at 37°C for 30 min. The amounts of TBARS were determined as described above.

2.8. Pulse radiolysis studies on reactions between EPC-K1 / hydroxyl radicals

For pulse radiolysis, a BF-5 electronic linear accelerator providing 5 MeV electron pulse with a duration of 2 μs was employed. The dosimetry of electron pulse was determined by 0.01 mol/l aqueous KSCN solution saturated with N_2O $\{G[(SCN)_2\cdot^-] = 6.0,\ \varepsilon_{480\ nm} = 7600\ dm^3\ mol^{-1}\ cm^{-1}\}$. The typical absorbed dose with one single pulse was 7 Gy.

Aqueous solutions of EPC-K1 were saturated with N_2O before pulse radiolysis. The transient spectra of intermediates formed by reactions between EPC-K1/$\cdot$OH were recorded. For kinetic study, aqueous solutions containing 0.01 mol/l KSCN and different concentrations of EPC-K1 were saturated with N_2O before pulse

radiolysis. The transient absorption at 480 nm was recorded. The rate constant of reaction between EPC-K1 and hydroxyl radicals was obtained by the competition kinetic method.

2.9. Pulse radiolysis studies on reactions between EPC-K1 / linoleic acid radicals

Lipid radicals were generated by reactions between ·OH/H· and aqueous solutions of linoleic acid (approx. 1 mmol/l) saturated with N_2O [16]. In EPC-K1/linoleic acid reaction mixtures, when the linoleic acid/EPC-K1 concentration ratio was higher than 20, most of ·OH (> 95%) would initially react with linoleic acid to form carbon-centered lipid radicals. Aqueous solutions of linoleic acid/EPC-K1 saturated with N_2O were irradiated and the transient spectra of EPC-K1 reacting with linoleic acid radicals were recorded. The rate constant of EPC-K1 reacting with linoleic acid radicals was calculated from the growth curve of the intermediate.

3. Results

3.1. Scavenging effect of EPC-K1 on hydroxyl radicals

EPC-K1 could scavenge hydroxyl radicals with an IC_{50} of 3.2 mmol/l (Fig. 1). Trolox was a poor scavenger of hydroxyl radicals which scavenged $33.9 \pm 5.7\%$ hydroxyl radicals at a concentration of 4 mmol/l. However, in comparison with vitamin C, EPC-K1 was only a moderate scavenger on hydroxyl radicals. Under the same conditions, 200 μmol/l of vitamin C scavenged up to 100% hydroxyl radicals generated in the reaction system.

3.2. Scavenging effect of EPC-K1 on alkyl radicals

Azo compound AAPH decomposes into carbon-centered alkyl radicals at a high rate [17]:

$$R - N = N - R \rightarrow 2R\cdot + N_2$$

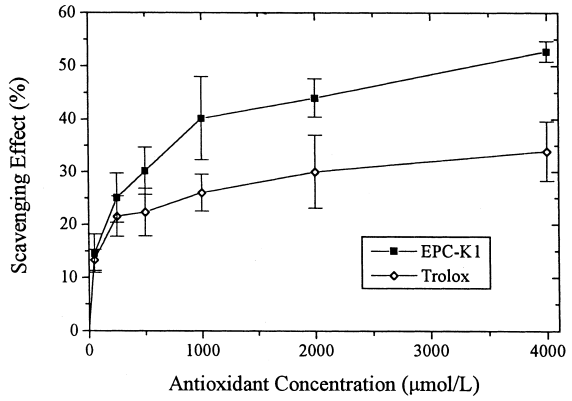

Fig. 1. Scavenging effects of EPC-K1 and Trolox on hydroxyl radicals. Data are mean ± S.D. of four samples.

EPC-K1 could scavenge AAPH-derived alkyl radicals with an IC_{50} of 200 μmol/l. Both Trolox and vitamin C were potent scavengers on these radicals with IC_{50} of approximately 60 μmol/l (Fig. 2).

3.3. Scavenging effect of EPC-K1 on lipid radicals

Both EPC-K1 and Trolox effectively decreased the signal intensity of the lipid radicals/POBN spin adduct generated by Fe-initiated lipid peroxidation in cerebellar neurons with IC_{50} of approximately 30 μmol/l (Fig. 3). In this reaction system, vitamin C could not scavenge lipid radicals.

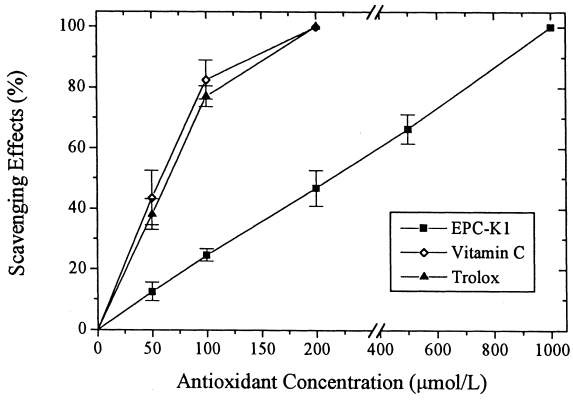

Fig. 2. Scavenging effects of EPC-K1, Trolox and vitamin C on alkyl radicals. Data are the mean ± S.D. of four samples.

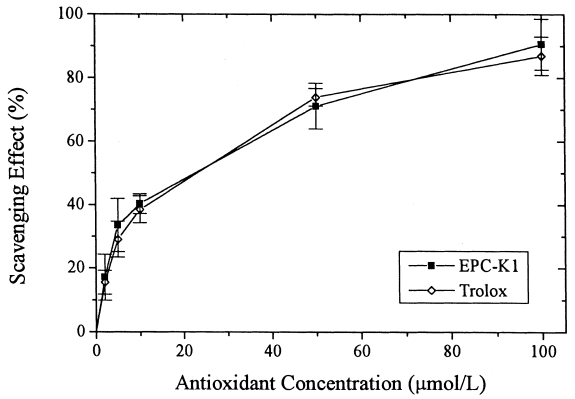

Fig. 3. Scavenging effects of EPC-K1 and Trolox on lipid free radicals. Data are the mean ± S.D. of four samples.

3.4. Inhibition effect of EPC-K1 on peroxyl-initiated lipid peroxidation

When oxygen exists, azo compounds AAPH (water-soluble initiator) and AMVN (lipid-soluble initiator) decompose into alkyl radicals, which react with molecular oxygen at high rates to form peroxyls:

$$R\cdot + O_2 \rightarrow ROO\cdot$$

In AAPH-initiated lipid peroxidation in brain submitochondrial particles, EPC-K1, as well as Trolox, inhibited the formation of TBARS with IC_{50} of approximately 25 μmol/l and was more effective than vitamin C (IC_{50} = 55 μmol/l) (Fig. 4a). In AMVN-initiated lipid peroxidation, EPC-K1 was a potent inhibitor with an IC_{50} of approximately 15 μmol/l and was more effective than Trolox (IC_{50} = 22 μmol/l) and vitamin C (IC_{50} = 50 μmol/l) (Fig. 4b).

3.5. Kinetic study of reaction between EPC-K1 and hydroxyl radicals

A series of reactions occur during electron irradiation in diluted aqueous solutions, and reactive species, such as hydroxyl radicals, hydrogen radicals, and aqueous electrons are formed:

$$H_2O \rightarrow H\cdot(0.55), \cdot OH(2.7), e^-_{aq}(2.7), H_3O^+, H_2O_2, H_2 \quad \text{(i)}$$

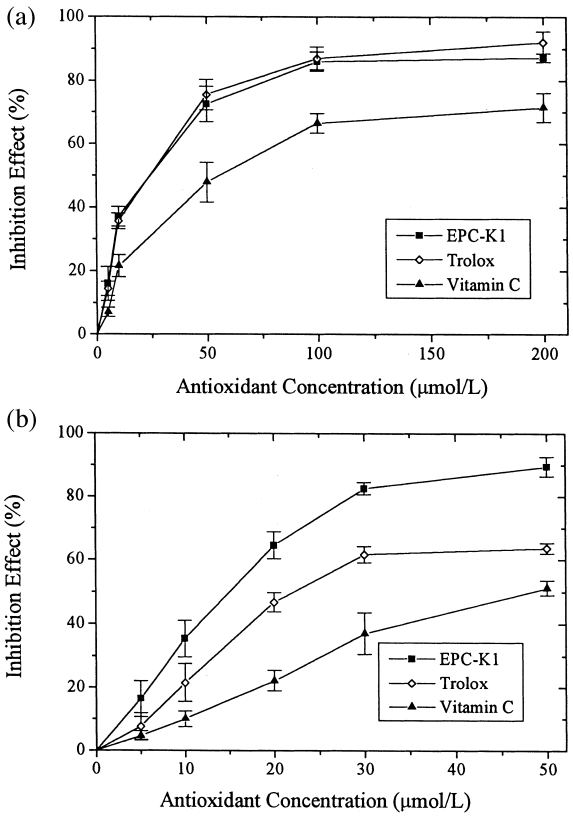

Fig. 4. (a) Inhibition effects of EPC-K1, Trolox and vitamin C on TBARS formation in AAPH-initiated lipid peroxidation. Data are the mean ± S.D. of four samples. (b) Inhibition effects of EPC-K1, Trolox and vitamin C on TBARS formation in AMVN-initiated lipid peroxidation. Data are the mean ± S.D. of four samples.

In N_2O-saturated solutions, e^-_{aq} converts to hydroxyl radicals quantitatively:

$$H_2O + N_2O + e^-_{aq} \rightarrow N_2 + OH^- + \cdot OH \quad k = 9 \times 10^9 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1} \quad \text{(ii)}$$

The transient spectrum of intermediates formed by EPC-K1 reacting with hydroxyl radicals was shown in Fig. 5. The maximum absorption was at 300 nm.

According to the following competition reactions:

$$\cdot OH + SCN^- \rightarrow OH^- + SCN\cdot \quad k = 1 \times 10^{10} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$$

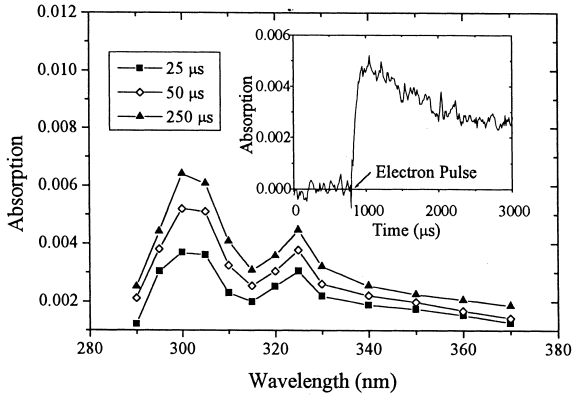

Fig. 5. Transient UV-visible spectrum of N_2O-saturated aqueous solution containing 0.5 mmol/l EPC-K1. The insert showed the decay of intermediates formed by a reaction between EPC-K1/·OH.

$$SCN\cdot + SCN^- \rightarrow (SCN)_2\cdot^- \quad \text{(iii)}$$

$$\cdot OH + \text{EPC-K1} \rightarrow \text{products} \quad \text{(iv)}$$

The following equation was obtained by the principle of competition kinetics:

$$OD_0/OD - 1 = (k_{\text{EPC-K1}}/k_{SCN^-}) \cdot([\text{EPC-K1}]/[SCN^-])$$

Here OD_0 and OD were the transient absorption at 480 nm in the absence and presence of EPC-K1, respectively. Plotting $OD_0/OD - 1$ against [EPC-K1]/[SCN^-] and employing the value 1×10^{10} dm^3 mol^{-1} s^{-1} for $k_{SCN^- \cdot OH}$, $k_{EPC\text{-}K1 \cdot OH} = 7.1 \times 10^8$ dm^3 mol^{-1} s^{-1} was calculated (Fig. 6).

3.6. Kinetic study of reaction between EPC-K1 and linoleic acid radicals

The transient spectrum of EPC-K1 reacting with linoleic acid radicals (L·) was shown in Fig. 7. The maximum absorption was at 300 nm, which was the same as the transient spectrum observed in EPC-K1/·OH reaction systems. These results suggested that the intermediates formed by reactions between EPC-K1/·OH and EPC-K1/L· were the same. These intermediates might be the transient products of hydrogen abstraction. From the growth curve of intermediates formed by EPC-K1 reacting with L·, the rate constant of

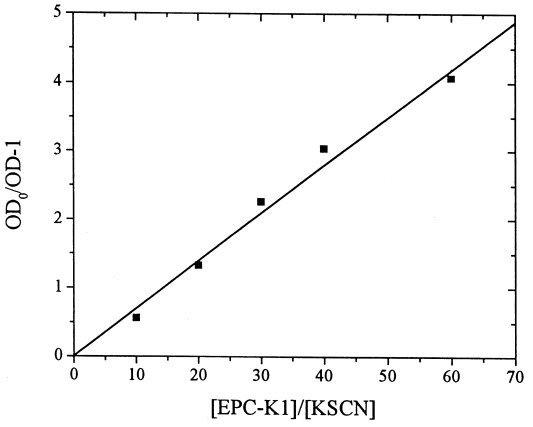

Fig. 6. Plot of competition kinetics of reactions between EPC-K1/·OH and KSCN/·OH.

2.8×10^6 dm^3 mol^{-1} s^{-1} was calculated. Because the transient absorption of Trolox reacting with linoleic acid radicals was very weak, the rate constant of Trolox reacting with linoleic acid was not calculated.

3.7. Effect of administration of EPC-K1 in vivo on the superoxide radical scavenging ability of rat brain

After i.p. administration of EPC-K1 for a week, the superoxide radical scavenging ability of rat brain significantly increased in comparison with control group. In EPC-K1-treated group, 34.5 ± 2.7% of superoxide radicals were scavenged with

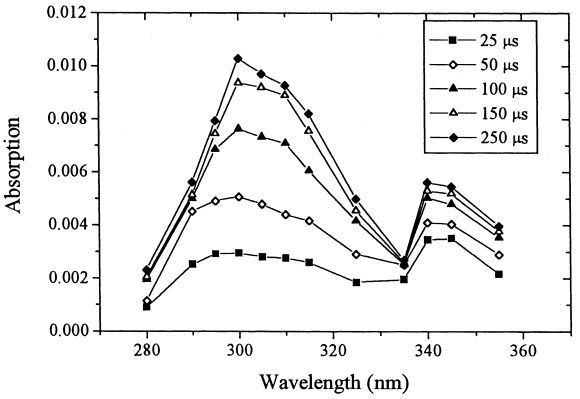

Fig. 7. Transient UV-visible spectrum of N_2O-saturated aqueous solution containing 1 mmol/l linoleic acid and 50 μmol/l EPC-K1.

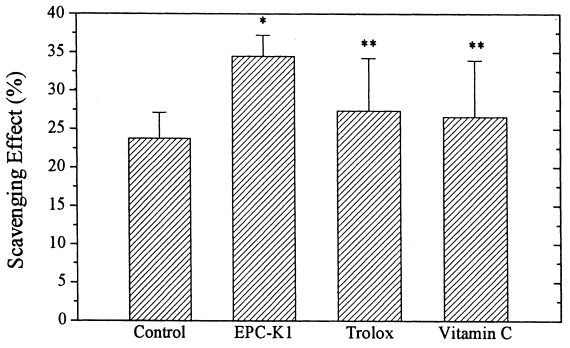

Fig. 8. Effect of administration of EPC-K1, Trolox and vitamin C on the superoxide radical scavenging ability of rat brain. Data are the mean ± S.D. of six samples. $^{*}P < 0.01$ in comparison with control; $^{**}P > 0.1$ in comparison with control.

5 mg/ml brain homogenate. In the control group, only $23.8 \pm 3.3\%$ of superoxide radicals were scavenged. Administration of Trolox or vitamin C at the same dose had no effect on the superoxide radical scavenging ability of rat brain (Fig. 8).

4. Discussion

EPC-K1, a phosphate ester derivative of vitamin C and vitamin E, showed different antioxidant capacity in comparison with vitamin C and Trolox. ESR spin trapping results revealed that EPC-K1 was a more effective scavenger of hydroxyl radicals than Trolox. It was the structural similarity with vitamin C that endowed EPC-K1 with good scavenging effects on hydroxyl radicals. Pulse radiolysis results showed that EPC-K1 reacted with hydroxyl radicals with a rate constant of 7.1×10^8 dm^3 mol^{-1} s^{-1}, which was less than that of vitamin C (10^{10} dm^3 mol^{-1} s^{-1}; [18]). This was consistent with the ESR spin trapping results that in comparison with vitamin C, EPC-K1 was only a moderate scavenger of hydroxyl radicals. The moderate scavenging effect of EPC-K1 on hydroxyl radicals might be partially due to its bulky phytyl side chain.

EPC-K1, as well as Trolox, could effectively decrease the ESR signal of POBN/lipid radicals spin adduct formed during Fe^{3+}/Fe^{2+}-initiated lipid peroxidation in cerebellar neurons. This was due to the direct scavenging on lipid radicals and/or the inhibition on Fe^{3+}/Fe^{2+}-initiated lipid peroxidation. In order to find out whether EPC-K1 could scavenge lipid radicals directly, pulse radiolysis technique was employed. The transient spectra suggested that EPC-K1 could react with linoleic acid radicals with a rate constant of 2.8×10^6 dm^3 mol^{-1} s^{-1}, while vitamin C could not scavenge lipid radicals. As a derivative of vitamin E, the hydrophobic phytyl side chain made EPC-K1 highly affinity with lipid radicals and thus EPC-K1 could act as an effective scavenger of them.

Several kinds of ROS including hydrophilic alkyl radical, peroxyl radicals and hydrophobic lipid radicals were involved in AAPH-initiated lipid peroxidation. The inhibition effect of EPC-K1 on TBARS formation showed its overall antioxidant properties. In order to investigate whether EPC-K1 and other antioxidants inhibited AAPH-initiated lipid peroxidation by scavenging AAPH-derived radicals, we employed the ESR spin trapping technique. The results showed that in comparison with vitamin C and Trolox, EPC-K1 was only a moderate scavenger on AAPH-derived alkyl radicals. Considering the potent inhibition effects of EPC-K1 on AAPH-initiated lipid peroxidation, we may conclude that EPC-K1 inhibited lipid peroxidation mainly by scavenging the secondary radicals formed during the chain reactions of lipid peroxidation. Potent scavenging effects of EPC-K1 on both hydrophobic and hydrophilic radicals made it an effective inhibitor on lipid peroxidation. Furthermore, we studied the inhibition effect of EPC-K1 on AMVN-initiated lipid peroxidation. Different from AAPH, which generates hydrophilic radicals, AMVN generates hydrophobic radicals. Our results indicated that EPC-K1 was also a potent inhibitor on AMVN initiated lipid peroxidation and was more effective than hydrophilic antioxidants vitamin C and Trolox.

The antioxidant active sites of vitamin C are the two enolic hydroxyl groups, while that of vitamin E is the phenolic hydroxyl group. In EPC-K1, the phenolic hydroxyl group and one of the enolic hydroxyl groups are converted to ester bonds and thus can not act as H donors, and the only active site is the enolic hydroxyl (Fig. 9).

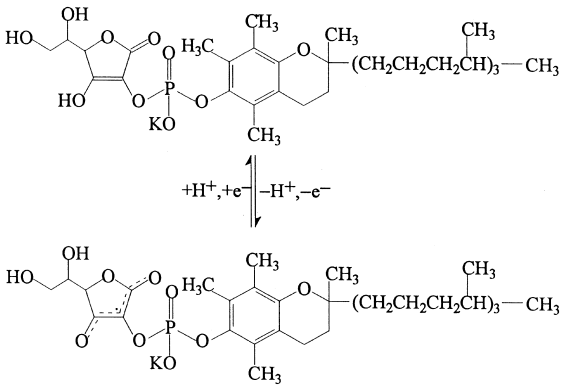

Fig. 9. Active site of EPC-K1.

This was also confirmed by the results of pulse radiolysis.

Superoxide radicals may be formed under certain pathological conditions, such as ischemia-reperfusion, so we studied the scavenging effect of rat brain on superoxide radicals after administration of EPC-K1. The results indicated that administration of EPC-K1 increased the ability of brain to scavenge superoxide radicals, while Trolox or vitamin C did not show this effect. This suggested that EPC-K1 might go through the blood-brain barrier.

In conclusion, EPC-K1 has better overall antioxidant properties in vitro and in vivo in comparison with vitamin C and Trolox. It was the special structure of EPC-K1 that endowed it with good ability of scavenging both hydrophilic radicals and hydrophobic lipid radicals at high rates. However, hydrophobic lipid radicals might diffuse across the membranes and play more important roles in oxidative damages than hydroxyl radicals and superoxide anions, while EPC-K1 is a potent scavenger on lipid radicals. For application, EPC-K1 is soluble in water and can be injected into body and then circulated all around the body. It also has a large hydrophobic substituent, which makes it possible to go through the blood vessel wall and thus enter the brain. This suggests that EPC-K1 may be used as a potential therapeutic agent on neurodegenerative diseases associated with oxidative damage.

Acknowledgements

We are indebted to the ESR Group of Institute of Biophysics, Academia Sinica for technical aids.

References

[1] B. Halliwell, Lancet 334 (1994) 721–724.
[2] J.T. Coyle, P. Puttfarcken, Science 262 (1993) 689–695.
[3] P. Jenner, Lancet 334 (1994) 796–798.
[4] M.F. Beal, Ann. Neurol. 38 (1995) 357–366.
[5] J. Marx, Science 273 (1996) 50–53.
[6] J. Hugon, Neurology 47 (1996) S251–S254.
[7] T. Ueda, T. Ueda, D. Armstrong, Ophthalmic Res. 28 (1996) 184–192.
[8] Y. Kuribayashi, H. Naritomi, M. Sasaki, T. Sawada, Arzneim.-Forsch. Drug Res. 44 (1994) 995–998.
[9] F. Block, M. Kunkel, K.H. Sontag, Brain Res. Bull. 36 (1995) 257–260.
[10] Q. Guo, B.L. Zhao, M.F. Li, S.R. Shen, W.J. Xin, Biochim. Biophys. Acta 1304 (1996) 210–222.
[11] J.A. North, A.A. Spector, G.R. Buettner, J. Biol. Chem. 267 (1992) 5743–5746.
[12] A.R. Kay, R.K.S. Wong, J. Neurosci. Methods 16 (1986) 227–238.
[13] A. Mori, J.K. Liu, X.Y. Wang, M. Kawai, Neurochem. Int. 24 (1994) 201–207.
[14] O. Inanami, Y. Watanabe, B. Syoto, M. Nakano, M. Tsuji, M. Kuwabara, Free Radic. Res. 29 (1998) 359–365.
[15] J.C.K. Lai, J.B. Clark, Methods Enzymol. 15 (1979) 51–60.
[16] T.P.A. Devasagayam, J.P. Kamat, H. Mohan, P.C. Kesavan, Biochim. Biophys. Acta 1282 (1996) 63–70.
[17] E. Niki, Methods Enzymol. 186 (1990) 100–108.
[18] M.G. Simic, Methods Enzymol. 186 (1990) 89–100.