Characterization of the Activity of L-Ascorbic Acid 2-[3,4-Dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-2H-1-benzopyran-6-yl-hydrogen Phosphate] Potassium Salt in Hydroxyl Radical Elimination

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The effect of L-ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-2H-1-benzopyran-6-yl-hydrogen phosphate] potassium salt (EPC-K₁) on hydroxyl radical ($\cdot\text{OH}$) elimination was studied using electron spin resonance (ESR) and spectrophotometric experiments. The addition of EPC-K₁ and $\cdot\text{OH}$ scavengers eliminated the $\cdot\text{OH}$ generated from $\text{Cu}^{2+}/\text{H}_2\text{O}_2$, $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ and $\text{H}_2\text{O}_2/\text{UV}$ -irradiation reaction systems. However, in competitive reactions using different concentrations of a spin-trap agent, the addition of the $\cdot\text{OH}$ scavenger altered the IC_{50} values, whereas the addition of EPC-K₁ and a metal chelater did not change the value in the $\text{Cu}^{2+}/\text{H}_2\text{O}_2$ and $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ reaction systems. The addition of EPC-K₁ and metal chelater changed the ESR signal for free Cu^{2+} . The spectrophotometric experiments confirmed that the addition of EPC-K₁ and metal chelater altered the absorption spectra due to CuCl_2 and FeSO_4 , whereas the $\cdot\text{OH}$ scavenger did not alter the spectra.

Therefore, it was demonstrated that EPC-K₁ has the ability both to scavenge $\cdot\text{OH}$ directly and to inhibit the generation of $\cdot\text{OH}$ by the chelation of Cu^{2+} and Fe^{2+} .

Key words ESR; hydroxyl radical; scavenging; L-ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-2H-1-benzopyran-6-yl-hydrogen phosphate] potassium salt

The hydroxyl radical ($\cdot\text{OH}$) generated in human tissue occasionally exerts unfavorable biological actions, such as lipid peroxidation and ischemia-reperfusion injury. It also plays an important role in tissue injury at inflammation sites in various diseases, including oral diseases.^{1,2)} It is well known that vitamin C and vitamin E have antioxidant effects and $\cdot\text{OH}$ scavenging ability. Recently, L-ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-2H-1-benzopyran-6-yl-hydrogen phosphate] potassium salt (EPC-K₁), a conjugation of vitamin C and vitamin E that is water-soluble and stable at room temperature, was developed as an $\cdot\text{OH}$ scavenger and antioxidant agent. Kuribayashi *et al.*³⁾ reported that EPC-K₁ inhibited lipid peroxidation and phospholipase A₂ activity in rat brain homogenates and human plasma, and suggested that these properties of EPC-K₁ might be useful in the treatment of ischemia-reperfusion. Yagi *et al.*⁴⁾ reported that the intravenous administration of EPC-K₁ had a strong protective effect on warm ischemic damage to liver grafts and recipient survival in swine. Previous studies also showed that EPC-K₁ had $\cdot\text{OH}$ scavenging and anti-lipid peroxidation properties.^{5–11)} Recently, Anzai *et al.*¹²⁾ reported that EPC-K₁ has potent $\cdot\text{OH}$ scavenging and iron chelating activity, and very weak reducing activity, and that these activities may work together to scavenge and reduce the $\cdot\text{OH}$, which may make EPC-K₁ beneficial *in vivo* by offering protection against $\cdot\text{OH}$ -related damage. In this study, we examined the ability of EPC-K₁ in detail.

Experimental

Materials EPC-K₁ was kindly provided by Dr. Kazumi Ogata, Senju Pharmaceutical (Japan). The chemical agents used for the generation of $\cdot\text{OH}$ were CuCl_2 , FeSO_4 and H_2O_2 . Ethylenediamine-*N,N,N',N'*-tetraacetic acid (EDTA) and diethylenetriamine-*N,N,N',N',N''*-pentaacetic acid (DETAPAC) were used for metal chelation, and dimethyl sulfoxide (DMSO) and ethanol were used as agents that scavenge $\cdot\text{OH}$. All of these chemicals were obtained from Wako Pure Chemical Co. (Japan). 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO), which is a trapping agent for radicals, was obtained from Dojin Chemicals (Japan).

Effects of EPC-K₁ on $\cdot\text{OH}$ Elimination The amounts of $\cdot\text{OH}$ generated from the $\text{CuCl}_2 + \text{H}_2\text{O}_2$ ($\text{Cu}^{2+}/\text{H}_2\text{O}_2$) and $\text{FeSO}_4 + \text{H}_2\text{O}_2$ ($\text{Fe}^{2+}/\text{H}_2\text{O}_2$) reaction systems were measured using the electron spin resonance (ESR) spin-trapping method.^{11,13,14)} The $\cdot\text{OH}$ eliminating ability of EPC-K₁ was analyzed by adding EPC-K₁ and an $\cdot\text{OH}$ scavenging agent to these reaction systems, then allowing them to react competitively with DMPO, the spin-trapping agent. The final concentration of DMPO in 200 μl of the $\text{Cu}^{2+}/\text{H}_2\text{O}_2$ reaction mixture (1 mM $\text{CuCl}_2/100$ mM H_2O_2) was adjusted to either 89.0 or 8.9 mM for this experiment. Various concentrations of EPC-K₁ and DMSO were added to the reaction mixture. In the $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ reaction system, the reaction mixture comprised 1.0 mM FeSO_4 solution and 100 mM H_2O_2 , along with 0.8 mM DETAPAC in 200 μl . The reaction was started by adding the H_2O_2 , and the $\cdot\text{OH}$ generated was measured with ESR after 50 s. Further, the amount of $\cdot\text{OH}$ generated from the reaction system after irradiation of 1 M H_2O_2 with ultraviolet light (UV, 254 nm) for 45 s was measured according to the method of Ueda *et al.*¹⁵⁾ The quantity of $\cdot\text{OH}$ was normalized relative to the standard signal intensity of the manganese oxide marker as $\cdot\text{OH}$ relative intensity = $\cdot\text{OH}$ signal intensity (first peak)/ Mn^{2+} marker intensity.¹¹⁾ The ESR spectra were measured using a JEOL JES FR-80 Radical Bio Sensor. The measurement conditions were as follows: microwave power, 8 mW; magnetic field, 335.4 $\pm$ 5 mT; sweep time, 2 min; modulation frequency, 100 kHz; and time constant, 0.3 s or 0.1 s (UV experiment).

Measurement of the ESR Signal for Cu^{2+} Since the ESR signals of EDTA-chelated Cu^{2+} and free Cu^{2+} are known to be different, in this experiment the influence of EPC-K₁ on Cu^{2+} was studied using ESR at a temperature of 77 K. For measurement of the free Cu^{2+} ESR signal, the final concentration of CuCl_2 was adjusted to 1 mM. Several concentrations of EDTA, DMSO and/or EPC-K₁ solutions were mixed with CuCl_2 , then their ESR signals were measured.

The measurement conditions were as follows: microwave power, 1.0 mW; magnetic field, 300.0 $\pm$ 100 mT; sweep time, 2 min; modulation frequency, 100 kHz; and time constant, 0.1 s. The coordinate bond of Cu^{2+} and EPC-K₁ was assessed from the ESR parameters obtained from the ESR signal. Fe^{2+} could not be measured as the diamagnetic.

Measurement of the Absorption Spectra of Cu and Fe The influence of EPC-K₁, DMSO and EDTA on the absorption spectra of CuCl_2 and FeSO_4 was studied. The final concentration of CuCl_2 or FeSO_4 was 1 mM in the total amount of 1200 μl . Alterations to the absorption spectrum were observed using a Shimadzu spectrophotometer UV-2200 over a 50 s period. The absorption range of Cu and Fe (from CuCl_2 and FeSO_4) was 202.4–327.4 nm and 208.4–511.0 nm, respectively. The influences of EPC-K₁, DMSO and EDTA (changes in CuCl_2 and FeSO_4 orientation) were determined in terms of the changes in instant absorbance.

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Table 1. IC_{50} (M) for Samples on Each Reaction System

	Cu^{2+}/H_2O_2 reaction system		Fe^{2+}/H_2O_2 reaction system		H_2O_2/UV -irradiation reaction system	
	DMPO 89.0 mM	DMPO 8.9 mM	DMPO 89.0 mM	DMPO 8.9 mM	DMPO 89.0 mM	
DMSO	8.9×10^{-2}	4.1×10^{-3}	1.0×10^{-2}	5.1×10^{-4}	DMSO	2.3×10^{-3}
EDTA	5.6×10^{-5}	5.6×10^{-5}	1.4×10^{-3}	1.4×10^{-3}	Ethanol	1.2×10^{-2}
EPC-K ₁	2.7×10^{-4}	1.2×10^{-4}	5.9×10^{-4}	4.0×10^{-4}	EPC-K ₁	2.8×10^{-2}

Fig. 1. Elimination Modes of $\cdot OH$ (%) by Adding Samples to the Cu^{2+}/H_2O_2 Reaction System

A) DMSO, B) EDTA, C) EPC-K₁. ●: 89.0 mM DMPO, ○: 8.9 mM DMPO. A hundred percent yield was given before the addition of sample.

Results

Elimination of $\cdot OH$ Generated from the Cu^{2+}/H_2O_2 Reaction System The $\cdot OH$ (DMPO- $\cdot OH$) was detected when DMPO was added to the reaction mixture. This adduct was confirmed as $\cdot OH$ because the hyperfine coupling constant was consistent with that given in a previous report ($a_N=a_H=1.49$ mT).¹⁶ Additions of EPC-K₁, DMSO and EDTA to the Cu^{2+}/H_2O_2 reaction system reduced the concentration of $\cdot OH$ (Fig. 1). Changing the DMPO concentration altered the DMPO- $\cdot OH$ level, showing that these agents are involved in a competitive reaction. When different concentrations of DMPO were used, the sigmoid curve was shifted laterally with the addition of DMSO, slightly with EPC-K₁, but

Fig. 2. Elimination Modes of $\cdot OH$ (%) by Adding Samples to the Fe^{2+}/H_2O_2 Reaction System

A) DMSO, B) EDTA, C) EPC-K₁. ●: 89.0 mM DMPO, ○: 8.9 mM DMPO. A hundred percent yield was given before the addition of sample.

not with EDTA. The IC_{50} (Table 1) was determined from the regression line obtained by the least-squares method, shown in Fig. 1. Remarkable change in IC_{50} values for EPC-K₁ or EDTA was observed by adding different concentrations of DMPO. However, the IC_{50} of DMSO varied with different concentrations of DMPO.

Elimination of $\cdot OH$ Generated from the Fe^{2+}/H_2O_2 Reaction System When EPC-K₁, DMSO and EDTA were added to the Fe^{2+}/H_2O_2 reaction system, the levels of DMPO- $\cdot OH$ adducts were reduced in a concentration-dependent manner (Fig. 2). The sigmoid curve shifted laterally with the addition of DMSO, but similar shifts were not observed with EPC-K₁ and EDTA. The IC_{50} values of EPC-K₁,

Fig. 3. ESR Signals from CuCl_2 with/without Each Solution

A) 1 mM CuCl_2 , B) 1 mM CuCl_2 + 7.5 mM EDTA, C) 1 mM CuCl_2 + 7.5 mM EPC- K_1 .

DMSO and EDTA are shown in Table 1. However, the IC_{50} of DMSO varied with different concentrations of DMPO.

Interaction between Cu^{2+} or Fe^{2+} and EPC- K_1 To confirm whether EPC- K_1 acted on the $\cdot\text{OH}$ generation system or on generated $\cdot\text{OH}$, we examined changes in the ESR signals for Cu^{2+} and changes in the absorption spectra of Cu^{2+} and Fe^{2+} . The alteration of the ESR signal for Cu^{2+} is shown in Fig. 3. The ESR signal obtained after the addition of EDTA was shifted to a higher field compared with the control. This shift in the signal indicated the chelation of EDTA with Cu^{2+} . In the EPC- K_1 experiment, the ESR signal for Cu^{2+} also changed, like EDTA, indicating EPC- K_1 also possesses chelating activity with Cu^{2+} . The effects of EPC- K_1 , EDTA and DMSO on the absorption spectra of CuCl_2 and FeSO_4 are shown in Fig. 4. The absorption spectra obtained by reacting CuCl_2 and FeSO_4 with EDTA shifted to the long-wavelength side compared with the spectrum of EDTA alone. The absorption spectra slightly shifted when EPC- K_1 was added to CuCl_2 and FeSO_4 . By contrast, no changes were seen with the addition of DMSO.

Elimination of $\cdot\text{OH}$ Generated from the H_2O_2 /UV-Irradiation Reaction System The $\cdot\text{OH}$ generated from the H_2O_2 /UV-irradiation reaction system was confirmed by the ESR spin-trapping method. When the $\cdot\text{OH}$ scavenger, DMSO or ethanol, was added to the reaction system, the amounts of $\cdot\text{OH}$ were decreased and new signals were observed (Fig. 5). The amount of $\cdot\text{OH}$ also decreased with the addition of EPC- K_1 . The level of DMPO- OH adducts was reduced in a concentration-dependent manner (Fig. 6). The IC_{50} values are shown in Table 1.

Discussion

It was reported that EPC- K_1 was composed of ascorbate and vitamin E, joined by a phosphodiester linkage, and had potent $\cdot\text{OH}$ scavenging ability and anti-lipid peroxidation ability.^{5–11)} Recently, Anzai *et al.*¹²⁾ reported the potency of EPC- K_1 using the ESR spin-trapping method and other methods. They have reported that $\cdot\text{OH}$ generated by the Fenton reaction ($\text{Fe}^{2+}/\text{H}_2\text{O}_2$) or by the UV irradiation of H_2O_2 was quenched efficiently and dose-dependently by EPC- K_1 . The spectrophotometric change of the mixture of EPC- K_1 and

Fig. 4. Spectrum Analyses

--- 1 mM CuCl_2 or 1 mM FeSO_4 , — sample alone, — 1 mM CuCl_2 or 1 mM FeSO_4 + sample.

Fig. 5. $\cdot\text{OH}$ Signals from the 1 M H_2O_2 /UV-Irradiation Reaction System with/without Each Solution

A) 1 M H_2O_2 /UV, B) 1 M H_2O_2 /UV + 3.5 M DMSO, C) 1 M H_2O_2 /UV + 4.3 M ethanol, D) 1 M H_2O_2 /UV + 6.25 mM EPC- K_1 . 89.0 mM DMPO was used in this experiment.

Fig. 6. Elimination Modes of $\cdot\text{OH}$ (%) by Adding Samples to the H_2O_2 /UV-Irradiation Reaction System with 89.0 mM DMPO

●: DMSO, ■: ethanol, ▲: EPC-K₁.

iron in water suggested that EPC-K₁ chelated the iron. As EPC-K₁ did not inhibit the peroxidation of linoleic acids caused by the reaction with an alkylperoxyl radical, EPC-K₁ did not have changing potency against the alkylperoxyl radical.¹²⁾

In previous studies, we reported⁷⁻⁹⁾ that the $\cdot\text{OH}$ was generated from the $\text{Cu}^{2+}/\text{H}_2\text{O}_2$ reaction system. However, details of the mechanism of action of EPC-K₁ with regard to $\text{Cu}^{2+}/\text{H}_2\text{O}_2$ have not been clarified. Therefore, we examined the potency of EPC-K₁ on the reaction systems of both $\text{Cu}^{2+}/\text{H}_2\text{O}_2$ and $\text{Fe}^{2+}/\text{H}_2\text{O}_2$. Further, Anzai *et al.*¹²⁾ did not examine the competitive reaction using different concentrations of spin-trap agent in the ESR experiment. In the present experiment, we examined the ability of EPC-K₁ to eliminate the $\cdot\text{OH}$ generated from three reaction systems: $\text{Cu}^{2+}/\text{H}_2\text{O}_2$, $\text{Fe}^{2+}/\text{H}_2\text{O}_2$, and H_2O_2 /UV-irradiation, by using ESR and the absorption spectra.

There are two possible mechanisms for the antioxidant action: inhibiting the generation of $\cdot\text{OH}$, or scavenging the generated $\cdot\text{OH}$. In the former action, the antioxidant may bind with transition metal ions that react with H_2O_2 and generate $\cdot\text{OH}$. The metal complex thus formed cannot further react with H_2O_2 to generate $\cdot\text{OH}$. In the latter, the lipid peroxidation induced by $\cdot\text{OH}$ is protected by direct scavenging of $\cdot\text{OH}$. Namely, $\cdot\text{OH}$ extracts a H atom from the lipid (LH), then the lipid radical ($\cdot\text{L}$) generated becomes a lipid peroxy radical ($\cdot\text{LOO}$) under the presence of O_2 . Further, the peroxy radical thus formed extracts H atom from the lipid to become hydroperoxide (LOOH) in the reaction of lipid peroxidation.¹⁷⁾

In the ESR spin-trapping method, the IC_{50} was not remarkably affected by the addition of EPC-K₁ or EDTA using different concentrations of DMPO in either the $\text{Cu}^{2+}/\text{H}_2\text{O}_2$ or $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ reaction system. The IC_{50} was, however, changed by the addition of the $\cdot\text{OH}$ scavenger, DMSO. The absorption spectra were altered by the addition of EPC-K₁ or EDTA, but not by the addition of DMSO. In addition, the

ESR signal of Cu^{2+} was altered by the addition of EPC-K₁ or EDTA. These results suggest that EPC-K₁ and EDTA may inhibit $\cdot\text{OH}$ generation by binding with the Cu^{2+} and Fe^{2+} . The complexes thus formed are considered to be unable to react with H_2O_2 to generate $\cdot\text{OH}$.

The concentration of $\cdot\text{OH}$ generated from the H_2O_2 /UV-irradiation reaction system was decreased by the addition of $\cdot\text{OH}$ scavengers, DMSO and ethanol. The concentration of the $\cdot\text{OH}$ generated from the UV irradiation reaction system was also decreased by the addition of EPC-K₁. In a pilot study, we confirmed that EPC-K₁ did not generate any radicals with UV irradiation (data not shown). These results suggest that EPC-K₁ also has a direct scavenging ability for $\cdot\text{OH}$. Our results agreed with the report of Anzai *et al.*¹²⁾

In conclusion, we confirmed that EPC-K₁ has the ability to inhibit the generation of $\cdot\text{OH}$ from the $\text{Cu}^{2+}/\text{H}_2\text{O}_2$ reaction system. We also reconfirmed the report of Anzai *et al.*¹²⁾ which stated that EPC-K₁ has the ability to inhibit the generation of $\cdot\text{OH}$ from $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ reaction system and to scavenge of $\cdot\text{OH}$ generated from the H_2O_2 /UV-irradiation reaction system. This report demonstrated that EPC-K₁ has two actions, namely, inhibiting the generation of $\cdot\text{OH}$ by the chelation of both Cu^{2+} and Fe^{2+} , and scavenging $\cdot\text{OH}$ directly.

These results suggest that EPC-K₁ might be effective for the treatment of various diseases in oral tissue. Studies for such application are currently under investigation in our group.

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