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

Takashi Tomita, Midori Kashima, and Yasuhisa Tsujimoto*

Department of Endodontics, Nihon University School of Dentistry at Matsudo, 870-1 Sakaecho, Nishi-2, Matsudo, Chiba 271–8587, Japan. Received August 19, 1999; accepted November 18, 1999

The effect of L-ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-2H-1-benzyopyran-6-yl-hydrogen phosphate] potassium salt (EPC-K1) on hydroxyl radical (·OH) elimination was studied using electron spin resonance (ESR) and spectrophotometric experiments. The addition of EPC-K1 and ·OH scavengers eliminated the ·OH generated from Cu2+/H2O2, Fe2+/H2O2, and H2O2/UV-irradiation reaction systems. However, in competitive reactions using different concentrations of a spin-trap agent, the addition of the ·OH scavenger altered the IC50 values, whereas the addition of EPC-K1 and a metal chelater did not change the value in the Cu2+/H2O2 and Fe2+/H2O2 reaction systems. The addition of EPC-K1 and metal chelater changed the ESR signal for free Cu2+. The spectrophotometric experiments confirmed that the addition of EPC-K1 and metal chelater altered the absorption spectra due to CuCl2 and FeSO4, whereas the ·OH scavenger did not alter the spectra.

Therefore, it was demonstrated that EPC-K1 has the ability both to scavenge ·OH directly and to inhibit the generation of ·OH by the chelation of Cu2+ and Fe2+.

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-benzyopyran-6-yl-hydrogen phosphate] potassium salt

Effects of EPC-K1 on ·OH Elimination

The amount of ·OH generated from the CuCl2+H2O2, (Cu2+/H2O2) and FeSO4+H2O2 (Fe2+/H2O2) reaction systems were measured using the electron spin resonance (ESR) spin-trapping method.11,13,14) The ·OH eliminating ability of EPC-K1 was analyzed by adding EPC-K1, and an ·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 Cu2+/H2O2 reaction mixture (1 μM CuCl2/100 μM H2O2) was adjusted to either 8.9 μM or 8.9 μM for this experiment. Various concentrations of EPC-K1 and DMSO were added to the reaction mixture. In the Fe2+/H2O2 reaction system, the reaction mixture comprised 1.0 mM FeSO4 solution and 100 μM H2O2, along with 0.8 μM DETAPAC in 200 μl. The reaction was started by adding the H2O2, and the ·OH generated was measured with ESR after 50 s. Further, the amount of ·OH scavenger from the reaction system after irradiation of 1 μM H2O2 with ultraviolet light (UV, 254 nm) for 45 s was measured according to the method of Ueda et al.15) The quantity of ·OH was normalized relative to the standard signal intensity of the manganese oxide marker as ·OH relative intensity = ·OH signal intensity (first peak)/Mn2+ marker intensity.11) The ESR spectra were measured using a JEOL JES-FR80 Radical Bio Sensor. The measurement conditions were as follows: microwave power, 8 mW; magnetic field, 335.4±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 Cu2+

Since the ESR signals of EDTA-chelated Cu2+ and free Cu2+ are known to be different, the ESR signal intensity of Cu2+ was measured using ESR at a temperature of 77 K. For measurement of the free Cu2+ ESR signal, the final concentration of CuCl2, was adjusted to 1 μM. Several concentrations of EDTA, DMSO, and/or EPC-K1 solutions were mixed with CuCl2, then their ESR signals were measured.

The measurement conditions were as follows: microwave power, 1.0 mW; magnetic field, 300.0±100 mT; sweep time, 2 min; modulation frequency, 100 kHz; and time constant, 0.1 s. The coordinate bond of Cu2+ and EPC-K1 was measured from the ESR parameters obtained from the ESR signal. Cu2+ 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 CuCl2 and FeSO4 was studied. The final concentration of CuCl2 or FeSO4 was 1 μM 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 CuCl2 and FeSO4) was 202.4—327.4 nm and 208.4—511.0 nm, respectively. The influences of EPC-K1, DMSO and EDTA (changes in CuCl2 and FeSO4 orientation) were determined in terms of the changes in the absorbance.

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* To whom correspondence should be addressed.
Results

Elimination of ·OH Generated from the Cu²⁺/H₂O₂ Reaction System  The ·OH (DMPO–OH) was detected when DMPO was added to the reaction mixture. This adduct was confirmed as ·OH because the hyperfine coupling constant was consistent with that given in a previous report ($aN = aH = 1.49$ mT). Additions of EPC-K₁, DMSO and EDTA to the Cu²⁺/H₂O₂ reaction system reduced the concentration of ·OH (Fig. 1). Changing the DMPO concentration altered the DMPO–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 not with EDTA. The IC₅₀ (Table 1) was determined from the regression line obtained by the least-squares method, shown in Fig. 1. Remarkable change in IC₅₀ values for EPC-K₁ or EDTA was observed by adding different concentrations of DMPO. However, the IC₅₀ of DMSO varied with different concentrations of DMPO.

Elimination of ·OH Generated from the Fe²⁺/H₂O₂ Reaction System  When EPC-K₁, DMSO and EDTA were added to the Fe²⁺/H₂O₂ reaction system, the levels of DMPO–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 IC₅₀ values of EPC-K₁,

<table>
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<tr>
<th>Table 1. IC₅₀ (m) for Samples on Each Reaction System</th>
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<tr>
<td><strong>Cu²⁺/H₂O₂ reaction system</strong></td>
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<tr>
<td>DMPO 89.0 mM</td>
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<tr>
<td>DMSO</td>
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<td>EDTA</td>
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<td>EPC-K₁</td>
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Fig. 1. Elimination Modes of ·OH (%) by Adding Samples to the Cu²⁺/H₂O₂ 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.

Fig. 2. Elimination Modes of ·OH (%) by Adding Samples to the Fe²⁺/H₂O₂ 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.
DMSO and EDTA are shown in Table 1. However, the IC₅₀ of DMSO varied with different concentrations of DMPO.

**Interaction between Cu²⁺ or Fe²⁺ and EPC-K₁** To confirm whether EPC-K₁ acted on the ·OH generation system or on generated ·OH, we examined changes in the ESR signals for Cu²⁺ and changes in the absorption spectra of Cu²⁺ and Fe²⁺. The alteration of the ESR signal for Cu²⁺ 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 Cu²⁺. In the EPC-K₁ experiment, the ESR signal for Cu²⁺ also changed, like EDTA, indicating EPC-K₁ also possesses chelating activity with Cu²⁺. The effects of EPC-K₁, EDTA and DMSO on the absorption spectra of CuCl₂ and FeSO₄ are shown in Fig. 4. The absorption spectra obtained by reacting CuCl₂ and FeSO₄ with EDTA shifted to the long-wavelength side compared with the spectrum of EDTA alone. The absorption spectra slightly shifted when EPC-K₁ was added to CuCl₂ and FeSO₄. By contrast, no changes were seen with the addition of DMSO.

**Elimination of ·OH Generated from the H₂O₂/UV-Irradiation Reaction System** The ·OH generated from the H₂O₂/UV-irradiation reaction system was confirmed by the ESR spin-trapping method. When the ·OH scavenger, DMSO or ethanol, was added to the reaction system, the amounts of ·OH were decreased and new signals were observed (Fig. 5). The amount of ·OH also decreased with the addition of EPC-K₁. The level of DMPO-OH adducts was reduced in a concentration-dependent manner (Fig. 6). The IC₅₀ values are shown in Table 1.

**Discussion**

It was reported that EPC-K₁ was composed of ascorbate and vitamin E, joined by a phosphodiester linkage, and had potent ·OH scavenging ability and anti-lipid peroxidation ability.⁵—¹¹ Recently, Anzai et al.¹² reported the potency of EPC-K₁ using the ESR spin-trapping method and other methods. They have reported that ·OH generated by the Fenton reaction (Fe²⁺/H₂O₂) or by the UV irradiation of H₂O₂ was quenched efficiently and dose-dependently by EPC-K₁. The spectrophotometric change of the mixture of EPC-K₁ and
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 reported¹⁻⁹) that the ·OH was generated from the Cu²⁺/H₂O₂ reaction system. However, details of the mechanism of action of EPC-K₁ with regard to Cu²⁺/H₂O₂ have not been clarified. Therefore, we examined the potency of EPC-K₁ on the reaction systems of both Cu²⁺/H₂O₂ and Fe²⁺/H₂O₂. Further, Anzai et al.¹³) 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 ·OH generated from three reaction systems: Cu²⁺/H₂O₂, Fe²⁺/H₂O₂, and H₂O₂/UV-irradiation, by using ESR and the absorption spectra.

There are two possible mechanisms for the antioxidant action: inhibiting the generation of ·OH, or scavenging the generated ·OH. In the former action, the antioxidant may bind with transition metal ions that react with H₂O₂ and generate ·OH. The metal complex thus formed cannot further react with H₂O₂ to generate ·OH. In the latter, the lipid peroxidation induced by ·OH is protected by direct scavenging of ·OH. Namely, ·OH extracts a H atom from the lipid (LH), then the lipid radical (·L) generated becomes a lipid peroxo radical (·LOO) under the presence of O₂. Further, the peroxo radical thus formed extracts H atom from the lipid to become hydroperoxide (LOOH) in the reaction of lipid peroxidation.¹⁵)

In the ESR spin-trapping method, the IC₅₀ was not remarkable affected by the addition of EPC-K₁ or EDTA using different concentrations of DMPO in either the Cu²⁺/H₂O₂ or Fe²⁺/H₂O₂ reaction system. The IC₅₀ was, however, changed by the addition of the ·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 Cu²⁺ was altered by the addition of EPC-K₁ or EDTA. These results suggest that EPC-K₁ and EDTA may inhibit ·OH generation by binding with the Cu²⁺ and Fe²⁺. The complexes thus formed are considered to be unable to react with H₂O₂ to generate ·OH.

The concentration of ·OH generated from the H₂O₂/UV-irradiation reaction system was decreased by the addition of ·OH scavengers, DMSO and ethanol. The concentration of the ·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 ·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 ·OH from the Cu²⁺/H₂O₂ 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 ·OH from Fe²⁺/H₂O₂ reaction system and to scavenge of ·OH generated from the H₂O₂/UV-irradiation reaction system. This report demonstrated that EPC-K₁ has two actions, namely, inhibiting the generation of ·OH by the chelation of both Cu²⁺ and Fe²⁺, and scavenging ·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.

References