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Direct radical scavenging by the bisbenzylisoquinoline alkaloid cepharanthine

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Abstract

Cepharanthine (Ceph) is known as a potent antiperoxidative agent. Recently, we characterized the antiperoxidative effects of Ceph [Biochim. Biophys. Acta 1426 (1999) 133]. However, it was not clear whether the antiperoxidative effect is really due to its direct radical scavenging activity. Therefore, we studied the interaction of Ceph with the hydroxyl radical (·OH) by the electron paramagnetic resonance (EPR) technique. Results showed that Ceph actually scavenged ·OH derived by the Fenton reaction. We also found that Ceph radicals were generated on interaction of Ceph with ·OH in neutral aqueous solution, but not in acidic solution, consistent with the pH-dependent anti-lipid peroxidation activity of Ceph. Hence, we concluded that anti-lipid peroxidation by Ceph is due to its direct radical scavenging activity.

Keywords: Cepharanthine; Antiperoxidant; Radical scavenging; Electron paramagnetic resonance; Lipid peroxidation

1. Introduction

The bisbenzylisoquinoline alkaloid cepharanthine (Ceph, chemical structure shown in Chart 1) has been known to have various biological effects, such as inhibitions of histamine release from mast cells [1], erythrocyte sickle cell formation [2] and platelet aggregation [3], and recovery from multidrug resistance [4]. Ceph is also reported to show antitumor activity [5], suppression of cytokine production [6], and induction of apoptosis [7].

In addition, Ceph has long been known to effectively inhibit lipid peroxidation [8–11], and this effect is sometimes thought to be associated with its versatile biological activities either directly or indirectly. We have reported that Ceph significantly inhibits Fe^{2+}/ADP-induced lipid peroxidation of both mitochondria and liposomes, and that it scavenges 1,1-diphenyl-2-picrylhidrazyl radicals and prevents formation of the 5,5′-dimethyl-1-pyrroline-*N*-oxide (DMPO) adduct with ·OH generated by the Fenton reaction in aqueous ethanol [11]. These results suggest that the antiperoxidative activity of Ceph is due to its radical trapping. However, as ethanol can scavenge radicals, it is not yet known whether Ceph directly traps radicals, or whether its anti-lipid peroxidation effect is due to trapping of Fe^{2+} added to generate reactive oxygen species (ROS). In fact, we did not clearly detect Ceph radicals generated by ROS in aqueous solution. In addition, it has long been thought that the antiperoxidative effect of Ceph is due to change in the membrane structure caused by Ceph or to direct radical scavenging [10].

For understanding the action mechanism of the versatile biological effects of Ceph, it is important to determine whether Ceph can directly scavenge ROS. Therefore, in this study, we examined the effect of Ceph on ROS by electron paramagnetic resonance (EPR) techniques.

2. Materials and methods

2.1. Materials

Ceph was provided by Kaken Shoyaku Co. (Tokyo, Japan). All the other reagents were of the highest grade commercially available.

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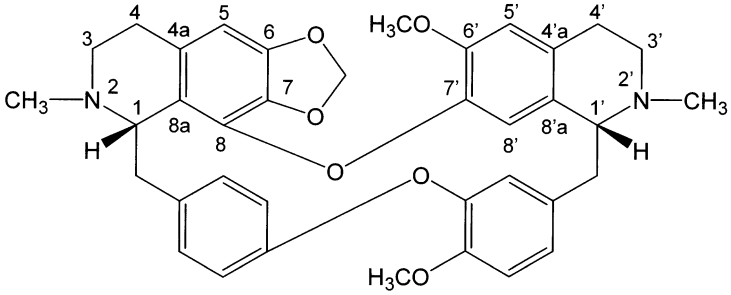

Chart 1. Chemical structure of Ceph.

2.2. Assay of lipid peroxidation of liposomes

Liposomes were prepared from egg yolk phosphatidylcholine by reverse-phase evaporation [12] in 10 mM Tris–HCl buffer (pH 7.4) containing 175 mM KCl. Liposomes (3 mM in terms of inorganic phosphate concentration) were incubated with various concentrations of Ceph for 5 min. Then 2,2′-azobis(2-amidinopropane)hydrochloride (AAPH) (final concentration, 5 mM) was added to initiate oxidation, and the mixture was incubated at 37 °C. After 5 h, the hydrophobic antioxidant butylated hydroxytoluene (final concentration, 4.5 mM) was added to terminate radical chain reactions. Amounts of lipid peroxides were expressed in terms of thiobarbituric acid-reactive substances (TBARS) from the absorbance at 532 nm with tetraethoxypropane as an external standard [13].

2.3. Absorption spectra

Ceph and $FeCl_3$ were dissolved in methanol. Optical absorption spectra of Ceph with or without Fe^{3+} were measured in a Shimadzu UV-1600 spectrophotometer.

2.4. Free radical analysis by EPR spectrometry

The free radicals of Ceph were examined in a JES-TE 300 EPR spectrometer (JEOL Co., Tokyo) equipped with a cavity and an aqueous quartz flat cell (JEOL) at the X-band (9.5 GHz) as described previously [14]. EPR spectra were analyzed in a computer (HP Apollo 9000 Series 400) with the software ESPRIT 432 (JEOL). Spectra were measured under the following conditions: power, 5 mW; center field, 3360 G; sweep width, 20 G; modulation frequency, 100 kHz; modulation width, 0.1 G; time constant, 0.03 s; sweep time, 1 min and temperature, 20 °C. Hyperfine coupling constants and simulation of spectra were determined with the computer program Winsim [15]. The radical scavenging rate constant was estimated from plots of the reciprocals of formation rates of ·OH/DMPO adduct against concentrations of Ceph as described previously [16].

2.5. UV irradiation experiment

Samples were irradiated with UV during passage through a quartz flat cell attached to the EPR cavity. The degassed (N_2 purge, 15 min) reaction mixture was slowly passed through the cell (flow rate approximately 1 ml/min) to minimize the depletion of Ceph. The irradiation source was 300-W xenon arc light (Ushio, Tokyo) [17].

2.6. Hydroxyl radical production in a fast-flow system

Because of the short lives of radical species, a fast-flow method [18] was adopted for both the Fenton [19] and horseradish peroxidase (HRP) system. Two reservoirs were used to supply solutions to the flat cell. One reservoir contained 5 mM Ceph and 1 M $FeSO_4$ in 10 mM phosphate buffer (pH 4.0) (50 ml), and the other contained 10.8 mM H_2O_2 in 0.2 M phosphate buffer (pH 11.2) (20 ml). Each of the freshly prepared solutions was introduced into an injection syringe, which was then attached to the syringe pump to force these solutions into the flat cell. Polyethylene tubing was used to connect the syringes and the flat cell. Ceph with Fe^{2+} solution at a rate of 135 ml/h and H_2O_2 solution at a rate of 15 ml/h were introduced into the flat cell separately, and mixed inside the cell to measure EPR spectra at 20 °C. The final concentrations of Ceph, Fe^{2+}, and H_2O_2 were 4.5 mM, 0.9 M, and 1.08 M, respectively, in phosphate buffer (pH 7.0).

3. Results and discussion

In this study, we studied whether radical scavenging ability is responsible for the potent antiperoxidative activity of Ceph. For this, we monitored the interaction between Ceph and ROS by EPR. Recently, we showed that Ceph significantly inhibits Fe^{2+}/ADP-induced lipid peroxidation of mitochondria and liposomes, its antiperoxidative effects being more potent than those of the well-known hydrophobic antioxidant α-tocopherol [11]. First, we examined the effect of Ceph on the peroxidation of liposomes induced

by the hydrophilic artificial radical generator AAPH. We used AAPH radicals instead of ROS generated by Fe^{2+}/ADP, as it is expected to be favorable for analysis of antiperoxidation by Ceph. As shown in Fig. 1, Ceph inhibited peroxidation of liposomes induced by AAPH concentration-dependently. The antiperoxidative effect of Ceph was significantly more than that of α-tocopherol, as in lipid peroxidation induced by Fe^{2+}/ADP [11]. Accordingly, Ceph inhibited lipid peroxidation induced by the artificial radicals, suggesting that its potent inhibitory effect on lipid peroxidation induced by Fe^{2+}/ADP is due to its radical scavenging activity.

Previously, we found in EPR studies that Ceph effectively scavenged hydroxyl radicals [11]. However, in that experiment, Ceph was dissolved in ethanol to increase its solubility. As ethanol is an ·OH scavenger, it was not thus clear whether the radical trapping observed was either due to Ceph or ethanol. Therefore, in this study, we dissolved Ceph in phosphate buffer, and examined whether it scavenges ROS such as ·OH.

We examined the effect of Ceph on the formation of the ·OH adduct with the radical trapping agent DMPO by measuring EPR signals in aqueous solution. The ·OH was generated from a mixture of Fe^{2+} and H_2O_2 (Fenton reaction). As shown in Fig. 2A and B, Ceph decreased the EPR signal intensity of the ·OH/DMPO adduct with increase in Ceph concentration, showing that Ceph scavenged ·OH in water. The decrease in signal intensity might, however, have been due to decrease in the concentration of iron by complex formation with Ceph. Therefore, we measured the absorption spectra (200–350 nm) of Ceph with or without Fe^{3+}. Ceph has a peak at 282 nm, whereas Fe^{3+} shows broad absorption without any distinct peak in methanol (Fig. 3). When Fe^{3+} was added to Ceph, no shift of the peak was observed, showing that Ceph did not form a complex with iron. Accordingly, decrease in EPR signals of the ·OH/DMPO adduct by Ceph was concluded to be due to its scavenging of ·OH.

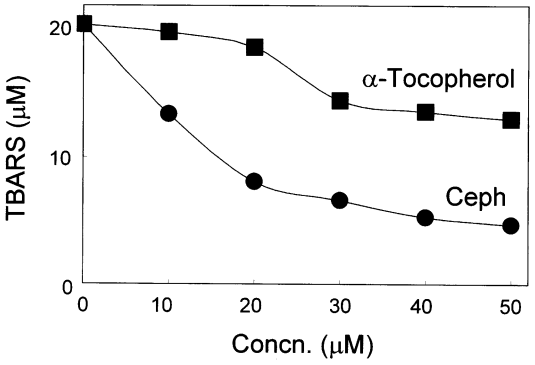

Fig. 1. Effects of Ceph and α-tocopherol on AAPH-induced lipid peroxidation of liposomes at pH 7.4. Various concentrations of Ceph or α-tocopherol dissolved in ethanol were incubated with liposomes (3 mM) for 5 min before induction of lipid peroxidation. Lipid peroxidation was induced by addition of 5 mM AAPH and terminated by addition of butylated hydroxytoluene (final concentration, 4.5 mM) after 5-h incubation at 37 °C. Amounts of lipid peroxides are expressed in terms of TBARS.

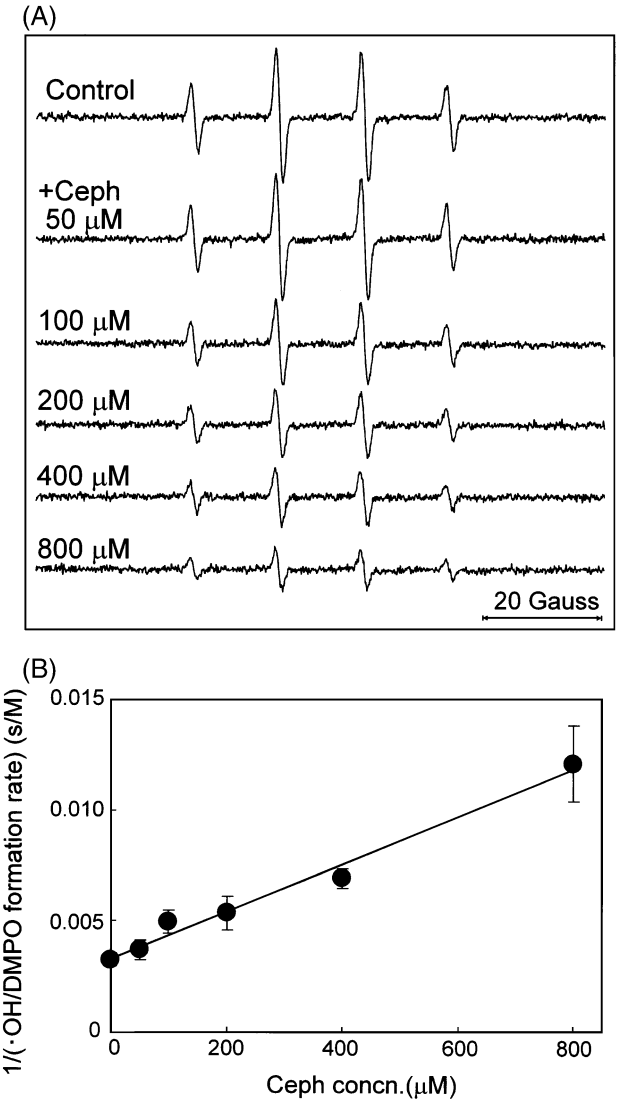

Fig. 2. Effects of various concentrations of Ceph on EPR spectra of the ·OH/DMPO adduct. (A) Spectra of the ·OH/DMPO adduct were obtained by addition of Fe^{2+} and H_2O_2 to a solution of 1 mM DMPO and various concentrations of Ceph dissolved in 0.1 M phosphate buffer (pH 7.4). The final concentrations of Fe^{2+} and H_2O_2 were 0.1 and 0.12 mM, respectively. EPR spectra were measured at 8 mW; modulation amplitude, 1.0 G; modulation frequency, 100 kHz; sweep time, 2 min; receiver gain, 790. (B) Plots are of reciprocals of the rate of formation of ·OH/DMPO adduct against the concentration of Ceph.

From the plot of the reciprocal of the formation rate of ·OH/DMPO adduct against concentration of Ceph shown in Fig. 2B, the apparent radical scavenging rate constant was determined as 1.39×10^{10} M^{-1} s^{-1}, which is greater than those of well-known antioxidants such as ascorbic acid (10^{10} M^{-1} s^{-1}), α-tocopherol (10^{10} M^{-1} s^{-1}) and butylated hydroxyltoluene (6×10^9 M^{-1} s^{-1}) [20], and is the same as that of the Ceph analog tetrandrine (1.4×10^{10} M^{-1} s^{-1}) [21]. The results indicate that Ceph is a potent ·OH

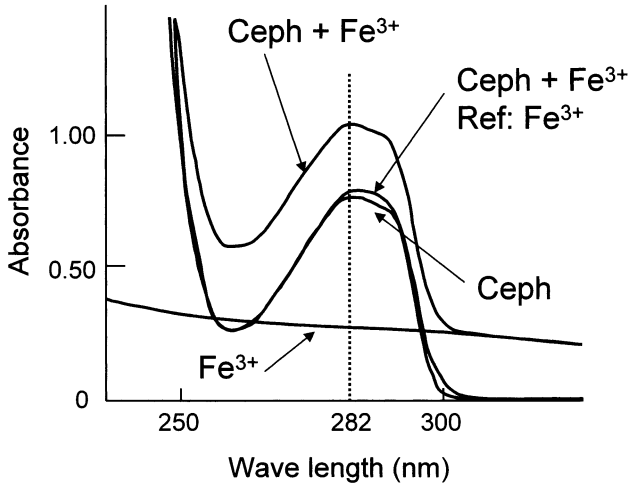

Fig. 3. Absorption spectra of Ceph with or without Fe^{3+}. Ceph (100 μM) and $FeCl_3$ (100 μM) were dissolved in methanol, and spectra were recorded with methanol as a reference, except for Ceph + Fe^{3+}, the spectrum of which was recorded with Fe^{3+} as a reference. Measurements were performed at room temperature.

radical scavenger. These results showed that the antiperoxidative effects of Ceph on Fe^{2+}-induced lipid peroxidation reported previously [8,9,11] were due to direct radical scavenging by Ceph.

On interaction of Ceph with ·OH, Ceph radicals should be formed. To confirm this, we examined the effect of UV irradiation on Ceph. Interestingly, an intense EPR signal appeared on exposure of Ceph solution to UV light (Fig. 4A), but it disappeared immediately when the UV light was turned off. This signal should be due to Ceph radicals. Possibly, the radicals were generated by split of the most reactive chemical bond of Ceph on UV irradiation. Next, generation of Ceph radicals on radical scavenging was examined. As the lifetime of Ceph radicals generated by UV irradiation was very short, it might not be possible to detect the radical signal by the general EPR technique. Therefore, we tried to detect the Ceph radical with the Fenton system at pH 7.0 using the fast-flow EPR method. As shown in Fig. 4B, a distinct EPR signal was observed at the same position as that observed on UV-irradiation on addition of Fe^{2+}/H_2O_2 to the solution of Ceph (see Fig. 4A). In contrast, no EPR signal was observed with Ceph alone, Ceph/H_2O_2 and Fe^{2+}/Ceph. From these results, we conclude that the EPR signal is due to the Ceph radical generated by scavenging ·OH, and that UV and ·OH attacked to the same site in Ceph.

As we reported previously that Ceph inhibited lipid peroxidation and scavenged DPPH radicals in solution at neutral pH, but not in acidic solution [11], we measured the EPR spectrum of Ceph at pH 4.0 in the presence of HRP and H_2O_2 [14], in which the target compound should be subjected to one-electron oxidation. We did not detect the signal of the Ceph radical (data not shown), indicating that Ceph does not react with radicals at acidic pH. This result is consistent with the pH-dependent antiperoxidation activity of Ceph [11]. This supports the radical scavenging mechanism we proposed previously, i.e., at neutral pH, the lone pair of N(2′) donates an electron to benzyl C(1′)-methine moiety, and as a result, ·H of the C(1′)-methine moiety could easily be eliminated by an external free radical. However, in acidic conditions, as the protonated N(2′) moiety is electron deficient, formation of conjugate between N(2′) and C(1′) is unlikely, resulting in loss of reactivity of the C(1′) moiety with a free radical [11].

In this work, we showed that Ceph directly scavenges radicals, and hence that its potent antiperoxidative effect is due to its radical scavenging activity. Our results should be useful for understanding the mechanisms of the various biological effects of Ceph.

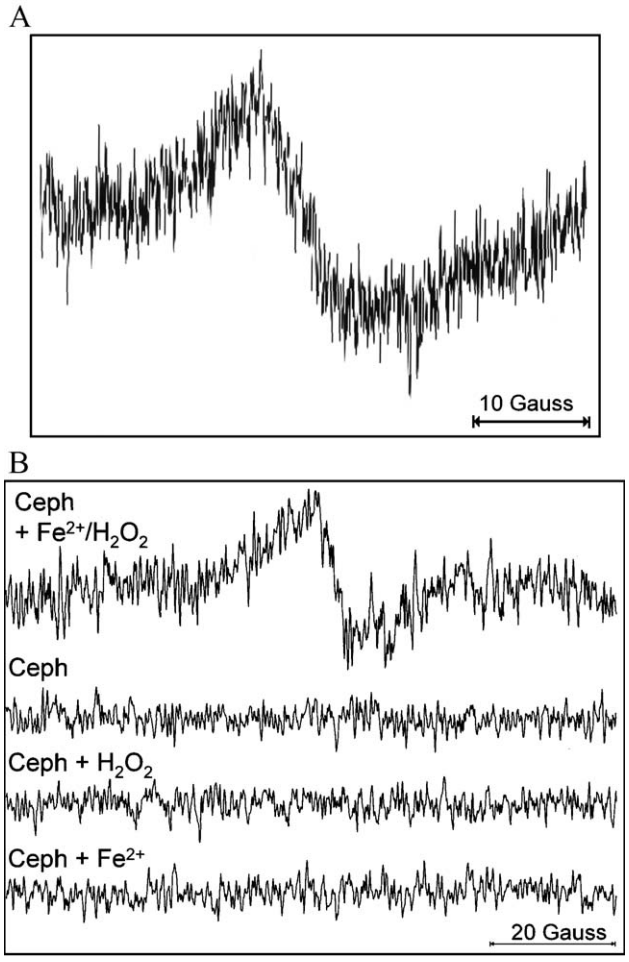

Fig. 4. EPR spectra of Ceph radicals generated by UV irradiation and reaction with ·OH produced by Fe^{2+}/H_2O_2. (A) EPR spectrum of Ceph irradiated with UV. An aqueous solution of 5 mM Ceph in 0.1 M phosphate buffer (pH 7.4) was exposed to UV light (300-W xenon arc light), and the EPR spectrum was recorded during irradiation. (B) Spectra of Ceph reacted with ·OH produced by Fe^{2+}/H_2O_2 in a fast-flow system. The final concentrations of Ceph, Fe^{2+}, and H_2O_2 were 4.5 mM, 0.9 M, and 1.08 M, respectively, in 0.2 M phosphate buffer (pH 7.0). EPR spectra were recorded at 20 mW; modulation, 1 G; amplitude, 2000; time constant, 0.01 s; accumulation, 16 times.

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