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Influences of Organic Solvents on CYPMPO-Electron Spin Resonance Spectra in *In Vitro* Radical Generating Systems

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ABSTRACT. Estimation of radical scavenging capacity of lipophilic antioxidants by electron spin resonance (ESR) *in vitro* is a challenging issue due to their poor solubility in aqueous radical generating and measuring systems. Water-miscible organic solvents are used for this purpose. A novel radical trapping agent, 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline N-oxide (CYPMPO), that has practical advantages over well-known trapping agents was synthesized. However, no available data for the influence of solvents in an ESR system that uses CYPMPO has been presented. The influences of six water-miscible organic solvents, acetonitrile (AcN), acetone, dimethyl sulfoxide (DMSO), ethanol, polyethylene glycol (PEG), and dimethoxyethane (DME), on ESR responses to Fenton Fe^{2+}/H_2O_2 OH· and hypoxanthine/xanthine oxidase superoxide generation systems *in vitro* were studied. Reduction of the ESR signal to CYPMPO-OH· adducts by 55.86 ± 5.95 and 83.17 ± 2.50% compared with the control was observed in the presence of AcN and acetone, respectively, at a final concentration of 5% (v/v). AcN of less than 1% had minimal effects. DMSO, ethanol, PEG and DME at 5% (v/v) strongly inhibited the ESR signals and/or caused derangement in the signal patterns. The six water-miscible solvents at 5% (v/v) had no influence on the ESR spectra of CYPMPO-superoxide adducts. From these results, AcN, at less than 1% (v/v), is a useful water-miscible organic solvent for assessing radical scavenging capacities of lipophilic compounds in the CYPMPO-Fenton Fe^{2+}/H_2O_2 OH· reaction system in an ESR assay. Any of the solvents used in the present study can be used in a hypoxanthine/xanthine oxidase superoxide generation system.

KEY WORDS: acetonitrile, CYPMPO, electron spin resonance, organic solvents.

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Reactive oxygen species (ROS) has been recognized as an important factor in a variety of disease processes and biological responses, including cardiovascular diseases, inflammatory conditions, defensive mechanisms, and cancer [3, 6, 8, 13, 16]. ROS affects biological components such as protein, nucleic acids and lipids to cause cell dysfunctions [9]. Antioxidants may suppress the initiation and propagation of the oxidative chain reaction and contribute to reducing the pathophysioloigcal states caused by ROS. The electron spin resonance (ESR) spin trapping method has been used for detection and characterization of ROS, such as superoxide and hydroxyl radicals, from biological, chemical and biochemical reactions [11, 17]. Estimation of radical scavenging capacity of lipophilic antioxidants by the in vitro ESR technique is a challenging issue due to their poor solubility in aqueous radical generating and measuring systems. Therefore, water-miscible organic solvents have been utilized to increase the solubility of lipophilic antioxidants in the *in vitro* ESR method. Several water-miscible organic solvents including methanol, ethanol, dimethyl sulfoxide (DMSO) and acetone, but not acetonitrile (AcN), had strong interference effects on a Fenton Fe^{2+}/H_2O_2 OH· generating system and significantly inhibited the signal intensity of ESR using 5,5-dimethyl N-oxide pyrroline (DMPO) [5]. DMPO has been the most frequently used spin trapping agent for identification and quantification of oxygen radicals [4]. However, its reaction rate with superoxide is slow, and the stability of the DMPO-superoxide spin adduct is poor (half life of the adduct: 1 min) [14]. Kamibayashi *et al.* [10] synthesized 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline N-oxide (CYPMPO, Fig. 1) as a novel radical trapping agent that has practical advantages over DMPO [14]. CYPMPO shows excellent spin

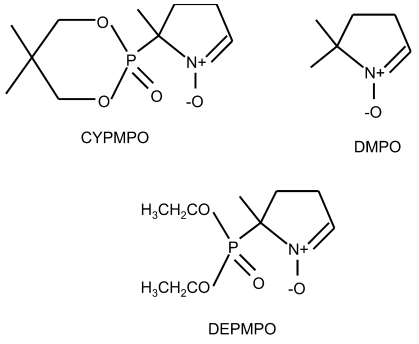

Fig. 1. Chemical structures of CYPMPO, DMPO and DEPMPO. CYPMPO, 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline N-oxide; DMPO, 5,5-dimethyl N-oxide pyrroline; DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-pyrroline N-oxide.

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trapping capabilities toward superoxide and hydroxyl radicals. Furthermore, ESR spectra in each adduct are clearly separated and readily identifiable. The ESR spectra of CYPMPO adducts remain stable for a long period [10, 12]. Moreover, CYPMPO has low cytotoxicity [14]. However, no precise data concerning the influence of organic solvents in an ESR system that uses CYPMPO is available. In the present study to identify suitable organic solvents for an ESR system that uses CYPMPO, the influences of six water-miscible organic solvents on ESR responses to Fenton Fe^{2+}/H_2O_2 OH· and hypoxanthine/xanthine oxidase superoxide generating systems were studied *in vitro*.

MATERIALS AND METHODS

Methods: The Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + 2OH\cdot$) was used to generate OH·. The spin-trapping reaction mixture for the Fenton reaction consisted of 0.1 m*l* of 10 mM CYPMPO, 0.1 m*l* of 0.10203 mM H_2O_2, 0.1 m*l* of 50 mM sodium phosphate buffer (pH 7.4) with and without organic solvents and 0.1 m*l* of 3 mM $FeSO_4$ mixed in this order. The reaction mixture was shaken well, and the ESR spectrum was recorded after 1 min after the addition of $FeSO_4$. A hypoxanthine/xanthine oxidase system was used to generate superoxide. The spin-trapping reaction mixture for the hypoxanthine/xanthine oxidase system consisted of 0.1 m*l* of 50 mM CYPMPO, 0.1 m*l* of 2 mM hypoxanthine, 0.1 m*l* of 0.4 U/m*l* xanthine oxidase and 0.1 m*l* of 50 mM potassium phosphate buffer containing 1 mM diethylenetriamine pentaacetic acid (DTPA; pH 7.4) with and without organic solvents. The reaction mixture was shaken well, and the ESR spectrum was recorded 1 min after shaking. ESR experiments were performed at room temperature using a JES-FA100 X-band spectrometer (JEOL Co., Ltd., Tokyo, Japan). The reaction mixture was placed in the ESR cavity using a 0.13-m*l* flat quartz glass cell (LC-12) for measurements. ESR measurements were performed under the following conditions: modulation frequency, 9.4 GHz; field modulation, 100 kHz; modulation amplitude, 0.1 mT; microwave power, 4 mW; center field, 335.944 mT; sweep-width, 5 mT; sweep time, 2 min; and time constant, 0.03 sec [15].

Chemicals: CYPMPO (gauche form) was synthesized according to the method of Kamibayashi *et al.* [10]. CYPMPO is commercially available from Radical Research Inc. (Tokyo, Japan). The other chemicals used were as follows: AcN, ethanol, $FeSO_4$, and dimethoxyethane (DME; Wako, Osaka, Japan); acetone, dimethyl sulfoxide (DMSO) and H_2O_2 (Kanto Chemical, Tokyo, Japan); hypoxanthine and DTPA (Sigma-Aldrich, St. Louis, MO, U.S.A.); polyethylene glycol (PEG; Nakalai Tesque, Kyoto, Japan); and xanthine oxidase (Roche Applied Science, Indianapolis, IN, U.S.A.).

Statistics: Results are expressed as means ± SEM. Statistical evaluation of the data was performed by ANOVA followed by Bonferroni's test. Results were considered significant when the *P* value was less than 0.05.

RESULTS

Influences of six water-miscible organic solvents (5% v/v) on ESR spectra of hydroxy radicals: The ESR spectra of CYPMPO-radical adducts in the Fenton Fe^{2+}/H_2O_2 OH· generation system with six water-miscible solvents are shown in Fig. 2. Typical 8-line spectra (8 time repetitive waves up and down from the middle in the spectra) corresponding to CYPMPO-OH· adducts were obtained [6]. Reduction of the ESR signal intensity by 55.86 ± 5.95% (n=4) and 83.17 ± 2.50% (n=4) compared with the control was observed in the presence of AcN and acetone, respectively, at a final concentration of 5% (v/v) in the reaction mixture. DMSO, ethanol, PEG and DME at 5% (v/v) strongly inhibited the ESR signals and/or caused derangement in the signal patterns. These data suggest that the water-miscible solvents used in the present study, except for AcN, are not suitable for measuring the OH· scavenging capacity of drugs using a CYPMPO-Fenton Fe^{2+}/H_2O_2 OH· reaction system.

Concentration-dependent effect of AcN on ESR spectra of hydroxy radicals: The influences of AcN at different concentrations ranging from 0.1 to 20% (v/v) on the Fenton reaction were examined (Fig. 3). Up to 1% (v/v) AcN showed only slight influence on the ESR spectra; the fold increases were 1.23 ± 0.18 for 0.1%, 0.93 ± 0.06 for 0.5% and 0.84 ± 0.05 for 1% (n=4, fold increase relative to the control). AcN at higher concentrations, 5, 10 and 20% (v/v), significantly and concentration-dependently inhibited the signals, with the fold increases being 0.50 ± 0.05, 0.30 ±

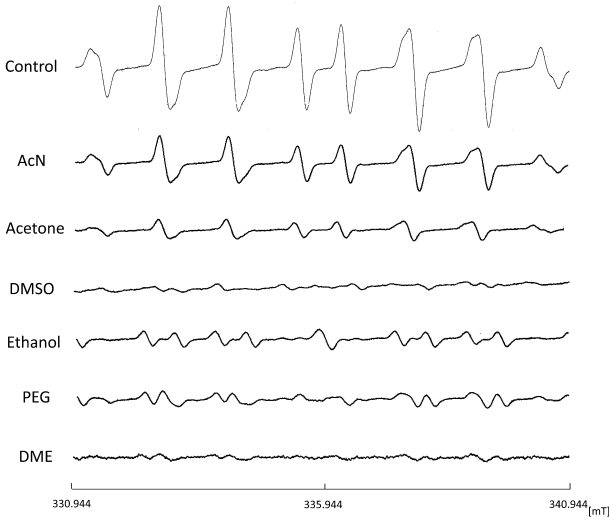

Fig. 2. Influences of the six water-miscible solvents (5% v/v) on ESR spectra of CYPMPO-OH· adducts. A fenton Fe^{2+}/H_2O_2 reaction system and CYPMPO were used as an OH· generation system and spin trapping agent, respectively. Reactions and measurements were performed at room temperature. The same results were obtained in 4 experiments repeated at different times. AcN, acetonitrile; DMSO, dimetyl sulfoxide; PEG, polyethylene glycol; DME, dimethoxyethane.

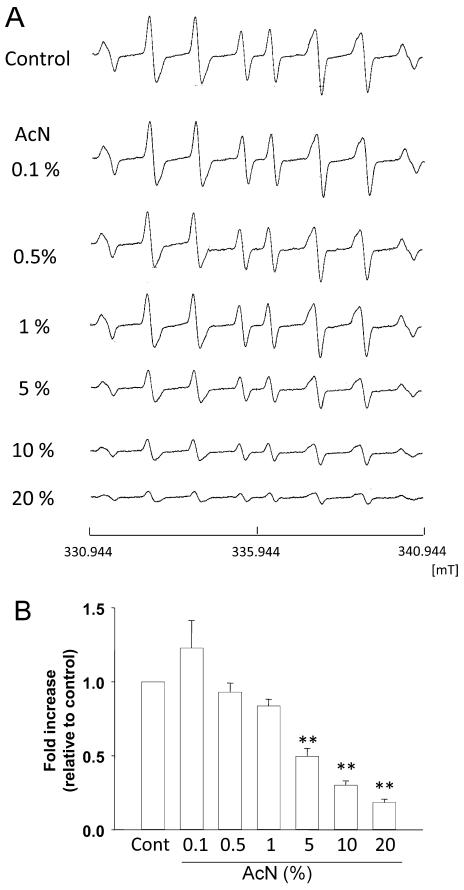

Fig. 3. Concentration-dependent effect of acetonitrile (AcN, 0.1 to 20% v/v) on ESR spectra of CYPMPO-OH· adducts. A. Representative ESR spectra. B. Cumulative results of 4 experiments. The relative ESR signal intensity normalized to an individual control recording was assessed by the size of the third wave. Results are shown as fold increase relative to the control. ** P<0.01 vs. cont.

0.03 and 0.18 ± 0.02, respectively (n=4, P<0.01, fold increase relative to the control). Less than 1% (v/v) AcN can be used for *in vitro* assessment of water-insoluble agents for radical scavenging capacities in a CYPMPO-Fenton Fe^{2+}/H_2O_2 OH· reaction system.

Influences of six water-miscible organic solvents (5% v/v) on ESR spectra of superoxide: The ESR spectra of CYPMPO-radical adducts in the hypoxanthine/xanthine oxidase superoxide generation system with six water-miscible solvents are shown in Fig. 4. Typical 8-line spectra corresponding to CYPMPO-superoxide adducts were obtained [10, 14]. All solvents used in the present study at a final concentration of 5% (v/v) had no influence on the ESR signals of the superoxide generation system. The ESR signal intensities in the presence of AcN, acetone, DMSO, ethanol, PEG and DME were 99.93 ± 6.37, 112.95 ± 3.94, 96.80 ± 6.30, 99.08 ± 7.80, 100.64 ± 3.64 and 107.48 ± 3.76, respectively (n=4, % relative to the control).

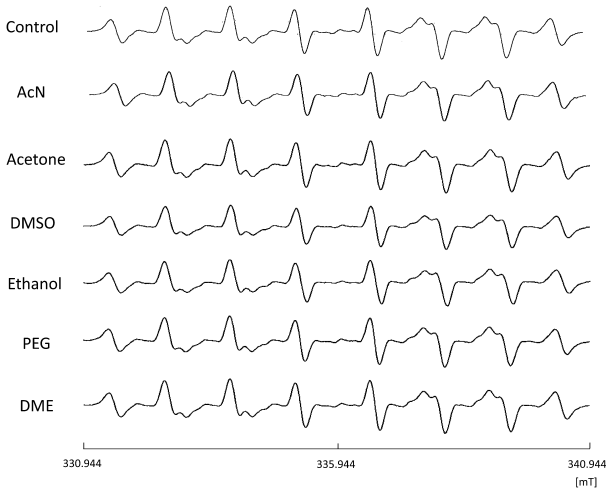

Fig. 4. Influences of the six water-miscible solvents (5% v/v) on ESR spectra of CYPMPO-superoxide adducts. A hypoxanthine/xanthine oxidase system and CYPMPO were used as a superoxide production system and spin trapping agent, respectively. Reactions and measurements were performed at room temperature. The same results were obtained in 4 experiments repeated at different times.

DISCUSSION

The ESR spin-trapping technique is the most reliable analytical method for detecting and characterizing ROS and radicals in biological systems [11, 17]. DMPO has been the most frequently used spin-trapping agent. However, DMPO has disadvantages in its reaction rate and stability of spin adducts [10]. DEPMPO is a better trapping agent than DMPO in terms of spin adduct stability [10]. However, it is a very hygroscopic oil, and its handling and purification require much effort [7]. On the other hand, CYPMPO is in a hygroscopic colorless crystalline state at room temperature. An aqueous solution of CYPMPO can be stored under ambient conditions for at least one month. The stability of the hydroxyl and superoxide adducts of CYPMPO is similar to that of DEPMPO [10]. Discrimination between the hydroxyl and superoxide adducts of CYPMPO is easier because the distance between the 4th and 5th lines in the ESR spectra is narrow in the CYPMPO-hydroxyl adducts, whereas it is wide in the CYPMPO-superoxide adducts (see Control in Figs. 2 and 4) [10, 14].

In the present study, the influences of six water-miscible organic solvents on the ESR signal intensity of CYPMPO-radical adducts were examined *in vitro*. Among the aprotic solvents, AcN and acetone inhibited the ESR signal intensity of the CYPMPO-Fenton Fe^{2+}/H_2O_2 OH· reaction system. However, typical 8-line spectra corresponding to CYPMPO-OH· adducts were observed in the presence of

AcN and acetone at 5% (v/v). AcN, at less than 1% (v/v), had practically no influence on the ESR signals of the CYPMPO-Fenton Fe^{2+}/H_2O_2 OH· system. The other aprotic solvents, DMSO, PEG and DME, caused strong inhibition and derangement of the ESR signal. It is known that DMSO traps hydroxyl radicals to form a stable and nonradical compound [2]. PEG shows a strong quenching effect on the ESR signal intensities of DMPO- and DEPMPO-OH· spin adducts [1]. These trapping activities of both solvents appeared in the CYPMPO-Fenton Fe^{2+}/H_2O_2 OH· reaction mixture used in the present study. A polar protic organic solvent, ethanol, has been shown to have a strong interference effect, like DMSO, on a DMPO-Fenton Fe^{2+}/H_2O_2 OH· generating system [3]. Ethanol at 5% (v/v) cannot be used in the CYPMPO system because it causes derangement of the ESR signal. These influences of water-miscible solvents on ESR signals of CYPMPO-radical adducts almost confirm the findings of a previous report concerning DMPO-OH· spin adducts [5].

In the case of ESR spectra of CYPMPO-superoxide adducts in the hypoxanthine/xanthine oxidase superoxide generation system, the six water-miscible organic solvents used in the present study had no influence on the typical 8-line spectra. All of the protic and aprotic organic solvents used in the present study seemed to be suitable for detection of the CYPMPO-superoxide adducts.

CYPMPO is superior to other spin-trapping agents, such as DMPO and DEPMPO, in nature [10, 12, 14]. Although the CYPMPO-ESR assay is a suitable method for screening antioxidants, there is insufficient information available about the influences of organic solvents on CYPMPO-induced ESR signals. The present study is the first to provide fundamental information on the effects of water-miscible organic solvents on CYPMPO-ESR signals.

In summary, AcN at a low concentration, less than 1% (v/v), is a useful water-miscible organic solvent for assessing the radical scavenging capacities of lipophilic compounds in the CYPMPO-Fenton Fe^{2+}/H_2O_2 OH· reaction system in an ESR assay. Furthermore, all the solvents used in the present study can be used for assessing CYPMPO-radical adducts in a hypoxanthine/xanthine oxidase superoxide generation system.

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