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Applicability of new spin trap agent, 2-diphenylphosphinoyl-2-methyl-3,4-dihydro-2*H*-pyrrole *N*-oxide, in biological system

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ABSTRACT

Electron spin resonance using spin-trapping is a useful technique for detecting direct reactive oxygen species, such as superoxide ($O_2^{\cdot-}$). However, the widely used spin trap 2,2-dimethyl-3,4-dihydro-2*H*-pyrrole *N*-oxide (DMPO) has several fundamental limitations in terms of half-life and stability. Recently, the new spin trap 2-diphenylphosphinoyl-2-methyl-3,4-dihydro-2*H*-pyrrole *N*-oxide (DPhPMPO) was developed by us. We evaluated the biological applicability of DPhPMPO to analyze $O_2^{\cdot-}$ in both cell-free and cellular systems. DPhPMPO had a larger rate constant for $O_2^{\cdot-}$ and formed more stable spin adducts for $O_2^{\cdot-}$ than DMPO in the xanthine/xanthine oxidase (X/XO) system. In the phorbol myristate acetate-activated neutrophil system, the detection potential of DPhPMPO for $O_2^{\cdot-}$ was significantly higher than that of DMPO (k_{DMPO} = 13.95 M^{-1} s^{-1}, $k_{DPhPMPO}$ = 42.4 M^{-1} s^{-1}). These results indicated that DPhPMPO is a potentially good candidate for trapping $O_2^{\cdot-}$ in a biological system.

Reactive oxygen species (ROS) have been implicated in several pathological conditions [1–4], and play a role in many normal physiological processes [5,6]. Particularly, superoxide anion ($O_2^{\cdot-}$) is most likely the initial ROS generated in cells. Many methods have been developed for assaying $O_2^{\cdot-}$ in chemical, biochemical, cellular, and *in vivo* systems. These include colorimetry, fluorometry, chemiluminescence assay, and electron spin resonance (ESR) spectroscopy [7]. Among these methods, ESR is the most direct and definitive technique in various systems. However, ESR is not sufficient for detecting short-lived species such as $O_2^{\cdot-}$ and a hydroxyl radical (·OH). Therefore, the spin-trapping technique has been used to stabilize highly reactive free radicals. The technique has become a valuable tool in the study of free radical processes occurring in various systems, including chemical, biochemical, and biological studies.

The spin trap 2,2-dimethyl-3,4-dihydro-2*H*-pyrrole *N*-oxide (DMPO) is widely used for detecting $O_2^{\cdot-}$. However, the $O_2^{\cdot-}$ adduct of DMPO (DMPO-OOH) has a relatively short life with a half-life of less than 1 min [8] and can spontaneously decompose or be metabolized to produce the hydroxyl adduct DMPO-OH in the presence of metal ions [9]. 2-(Diethoxyphosphinoyl)-2-methyl-3,4-dihydro-2*H*-pyrrole *N*-oxide (DEPMPO) is preferred due to its higher sensitivity and stability in cell-free and cellular systems [10,11]. It was reported that the $O_2^{\cdot-}$ adduct of DEPMPO (DEPMPO-OOH) does not decompose to the hydroxyl adduct and the half-life of DEPMPO-OOH is 14.8 min, more than 10 times higher than that of DMPO-OOH [12,13]. Recently, the new spin trap 2-diphenylphosphinoyl-2-methyl-3,4-dihydro-2*H*-pyrrole *N*-oxide (DPhPMPO) containing diphenylphosphinoyl was developed by our group. The spin-trapping rate constant of DPhPMPO for $O_2^{\cdot-}$ and ·OH is higher than that of DMPO. However, these previous studies have been carried out in cell-free systems such as the Fenton (H_2O_2–$FeCl_3$) and xanthine/xanthine oxidase (X/XO) systems [14,15]. Presently, there is a lack of evaluation of its ability to detect cell-generated $O_2^{\cdot-}$.

In order to use this new spin trap effectively, it is essential to understand its biological interactions. Therefore, the aim of this study was to evaluate the ability of DPhPMPO in trapping $O_2^{\cdot-}$ from neutrophils activated by phorbol myristate acetate (PMA) and to extend the use of DPhPMPO for quantifying $O_2^{\cdot-}$ in a cellular system. In this study, we evaluated the use of the new spin trap DPhPMPO for quantitative measurement of $O_2^{\cdot-}$ in enzymatic and biological systems.

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Materials and methods

Materials. DPhPMPO and DMPO were provided by Dojindo laboratory (Kumamoto, Japan) and used without additional treatment. The structures of the two $O_2^{\cdot-}$ spin adducts are shown in Fig. 1. Diethylenetriamine pentaactic acid (DTPA) was purchased from Dojindo. XO was purchased from Roche (Basel, Switzerland). PMA, superoxide dismutase (SOD), and xanthine were purchased from Sigma Chemical (St. Loius, MO, USA). Every buffer and solutions for the reaction mixtures was treated with Chelex 100 resin (Bio-Rad Laboratories, Richmond, CA, USA) before use to remove trace metals.

Isolation of neutrophils. Human neutrophils were isolated from blood of healthy volunteers, as previously described [16], using a commercial gradient of Ficoll–Hypaque. Then, the neutrophils were suspended in Krebs Ringer phosphate. The number of neutrophils was adjusted to 10^7/ml, and the cells were stored on melting ice.

Spin-trapping in xanthine/xanthine oxidase system. The reaction mixture was incubated with 0.1 mg/ml xanthine in 100 mM sodium phosphate buffer (SPB, pH 7.4) containing 0.5 mM DTPA in the presence of 25 mM spin trap. XO (0.1 U/ml) was added last to the mixture to start the reaction. The spectra were obtained at 1 min after starting the reaction. For decay constant measurements, 1 min after the addition of XO, SOD (500 U/ml) was added to the reaction mixture. ESR spectra were recorded at room temperature on a JES-TE 200 ESR spectrometer (JEOL, Tokyo, Japan) under the following conditions: modulation frequency, 100 kHz; modulation amplitude, 0.03 mT; scanning field, 335.3 ± 5 mT; receiver gain, 300–500; response time, 0.3 s; sweep time, 2 min; microwave power, 20 mW; and microwave frequency, 9.43 GHz.

Kinetic studies of $O_2^{\cdot-}$ trapping. The kinetic competition method was used to determine the rate constant for $O_2^{\cdot-}$ trapping by DPhPMPO employing DMPO as a competitive inhibitor, as previously described [17]. $O_2^{\cdot-}$ was generated in SPB using the X/XO system. For kinetic experiments, a fixed DPhPMPO concentration of 25 mM was chosen, and the DMPO concentration was varied from 0 to 25 mM. XO was added last to the mixture to start the reaction. The spectra were obtained at 1 min after starting the reaction. The relative abundance of the two components was calculated from the ratio of the peak height of the first peak to the left of DPhPMPO to the peak height of the first peak to the left of DMPO. A plot of the relative abundance of the spin adduct against the initial concentration of the spin afforded a line with the slope $k_{DPhPMPO}/k_{DMPO}$.

Detection of cell-generated $O_2^{\cdot-}$ by ESR. PMA is a phorbol ester that directly activates protein kinase C (PKC). In neutrophils, PMA has been reported to induce redistribution of PKC, phosphorylation of a number of different proteins, and activation of phagocyte NADPH oxidase [18]. PMA was used in the present study to stimulate $O_2^{\cdot-}$ generation in neutrophils. Each ESR sample contained 25 mM spin trap, 1×10^6 neutrophils/ml, 0.5 mM DTPA, and 5 ng/ml PMA, and the samples ware preincubated in a water bath at 37 °C for 8 min. ESR spectra were recorded at room temperature on a JES TE 200 ESR spectrometer under the following conditions: modulation frequency, 100 kHz; modulation amplitude, 0.03 mT; scanning field, 335.3 ± 5 mT; receiver gain, 300–500; response time, 0.3 s; sweep time, 2 min; microwave power, 20 mW; and microwave frequency, 9.43 GHz.

Cytochrome c reduction assay. The ferricytochrome *c* reduction rate of $O_2^{\cdot-}$ released from neutrophils was measured as reported previously at 540 and 550 nm using a Hitachi 557 double wavelength spectrometer equipped with a thermostat-controlled cell. The reaction mixture contained 1×10^7 neutrophils, 1 mM ferricytochrome *c* and 0.002 mg/ml PMA in Kreb's Ringer phosphate with or without various concentrations of DMPO or DPhPMPO. Spontaneous $O_2^{\cdot-}$ release was measured for 10 min at 37 °C. PMA was added to the reaction mixture and ferricytochrome *c* reduction was observed for 10 min. Ferricytochrome *c* reduction was completely terminated by adding 500 U/ml Cu, Zn-SOD. The amount of reduced cytochrome *c* was calculated using a molar absorption coefficient of 19.1×10^3. The initial rate of $O_2^{\cdot-}$ release was expressed as nanomoles of cytochrome *c* reduced/min/10^7 cells. We measured the 50% inhibitory concentration by adding various concentrations of DMPO or DPhPMPO in the $O_2^{\cdot-}$ generation system for kinetic studies.

Statistical analysis. Experiments were repeated at different days, and data were expressed as means ± SD. Two-tailed Student's *t*-test was conducted with SPSS, with a standard cut-off of $p < 0.05$.

Results

Trapping of $O_2^{\cdot-}$ in xanthine/xanthine oxidase system

We first carried out experiments to compare the $O_2^{\cdot-}$ trapping abilities of the two spin traps in the X/XO system under exactly the same experimental conditions. $O_2^{\cdot-}$ was generated by a 0.1 mg/mL xanthine and 0.1 U/ml XO system in SPB. Fig. 1A and B shows the typical ESR spectra of $O_2^{\cdot-}$ adducts of the spin traps DMPO and DPhPMPO. These ESR spectra were consistent with previously reported DMPO [19] and DPhPMPO-OOH [14] spectra having a hyperfine coupling constant of $a_N = 1.43$ mT, $a_H^\beta = 1.15$ mT,

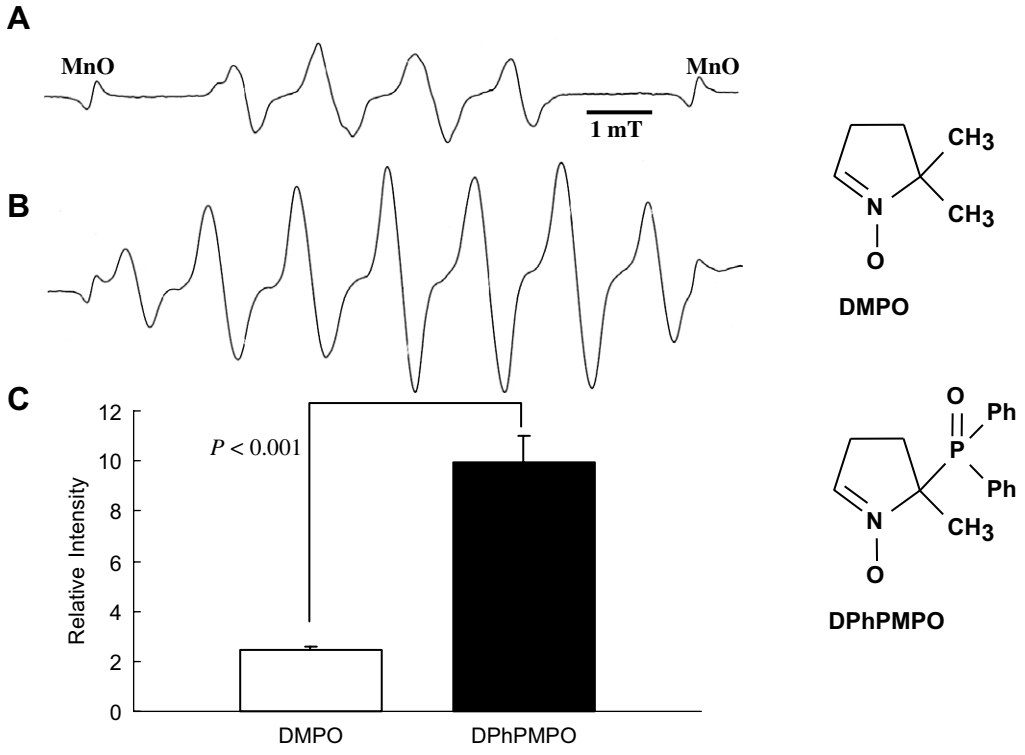

Fig. 1. Representative ESR spectra of $O_2^{\cdot-}$ spin adducts for DMPO (A) and DPhPMPO (B), and the intensity of $O_2^{\cdot-}$ spin adducts for DMPO and DPhPMPO (C) in the X/XO system. The bar graph reflects the peak-to-peak amplitude of the most intense resonance in the spectrum relative to the amplitude of manganese oxide. Data are expressed as means ± SD, $n = 3$.

a_H^{γ} = 0.13 mT for DMPO-OOH, a_N = 1.26 mT, a_H^{β} = 1.10 mT, a_P = 3.90 mT for DPhPMPO-OOH. These results indicate that the detection potential of DPhPMPO for $O_2^{\cdot-}$ was significantly higher than that of DMPO. The formation of spin adducts via $O_2^{\cdot-}$ trapping was confirmed in the experiments where SOD was added before XO, in which no ESR signals were observed (data not shown).

Spin-trapping of $O_2^{\cdot-}$: kinetics of spin-trapping

The kinetic competition method, with DMPO as a competitive scavenger, was used to determine the rate constant for the trapping of $O_2^{\cdot-}$ on DPhPMPO (Scheme 1). From the X/XO system containing both DMPO and DPhPMPO, typical ESR signals due to two $O_2^{\cdot-}$ adducts were observed (Fig. 2A). Signals were assigned by comparing those obtained from the X/XO system using DMPO and DPhPMPO. The relative rate constants for the $O_2^{\cdot-}$ of DPhPMPO and DMPO ($k_{DPhPMPO}/k_{DMPO}$) can be calculated from the slope of the straight lines obtained by plotting the relative abundance of two spin adducts ($R_{DPhPMPO}/R_{DMPO}$) against the ratio of the initial concentration ([DPhPMPO]/[DMPO]), as shown in Fig. 2B, with the slope equal to 3.21 and the coefficient equal to 0.99. Thus, the use of k_{DMPO} = 15 M^{-1} s^{-1} [9] led to $k_{DPhPMPO}$ = 48 M^{-1} s^{-1}. This data was also confirmed by cytochrome *c* reduction assay via the measurement of inhibitory capacity similar to the ESR study (k_{DMPO} = 15.8 M^{-1} s^{-1} [9] led to $k_{DPhPMPO}$ = 56.4 M^{-1} s^{-1} as determined by cytochrome *c* reduction assay).

Measurements of half-lives of DMPO-OOH and DPhPMPO-OOH adducts

To measure the half-lives of the DMPO-OOH and DPhPMPO-OOH adducts in the X/XO system, $O_2^{\cdot-}$ generation was initiated by adding XO and stopped by adding SOD. The kinetic constants of DMPO-OOH and DPhPMPO-OOH were 0.45 ± 0.03 min^{-1} and 0.11 ± 0.007 min^{-1}, respectively. Therefore, the obtained half-lives of DMPO-OOH and DPhPMPO-OOH were 1.5 ± 0.1 min and 6.2 ± 0.36 min, respectively.

Detection of cell-generated $O_2^{\cdot-}$

PMA is known to be a strong activator of PKC and activates the NADPH oxidase-derived oxidant burst of neutrophils. Using the spin traps DMPO and DPhPMPO, we analyzed ESR signals from neutrophils activated by PMA. Spectra of the spin trap adducts in the cellular system are shown in Fig. 4A and B. The observed spectrum of DMPO was the composite of the two species, DMPO-OOH and DMPO-OH. It has been shown that cellular components, such as glutathione (GSH)/GSH peroxidase, rapidly reduce DMPO-OOH to its hydroxyl adducts [20]. Therefore, It was suggested that DMPO trapped $O_2^{\cdot-}$ and that DMPO-OOH was reduced to DMPO-OH by neutrophils. The spectrum of DPhPMPO was confirmed as a $O_2^{\cdot-}$ spin adduct by its hyperfine coupling constant similar to that in the X/XO system. We compared the relative intensities of DMPO and DPhPMPO in Fig. 4C. These results indicate that the detection

$$O_2^{\cdot-} + \text{DPhPMPO} \xrightarrow{k_{DPhPMPO}} \text{DPhPMPO-OOH} \quad (1)$$

$$O_2^{\cdot-} + \text{DMPO} \xrightarrow{k_{DPhPMPO}} \text{DMPO-OOH} \quad (2)$$

$$\frac{R_{DPhPMPO}}{R_{DMPO}} = \frac{P_{DPhPMPO\text{-}OOH}}{P_{DMPO\text{-}OOH}} = \frac{k_{DPhPMPO}[\text{DPhPMPO}]}{k_{DMPO}[\text{DMPO}]} \quad (3)$$

Scheme 1. Reaction scheme of DPhPMPO and DMPO for kinetic study.

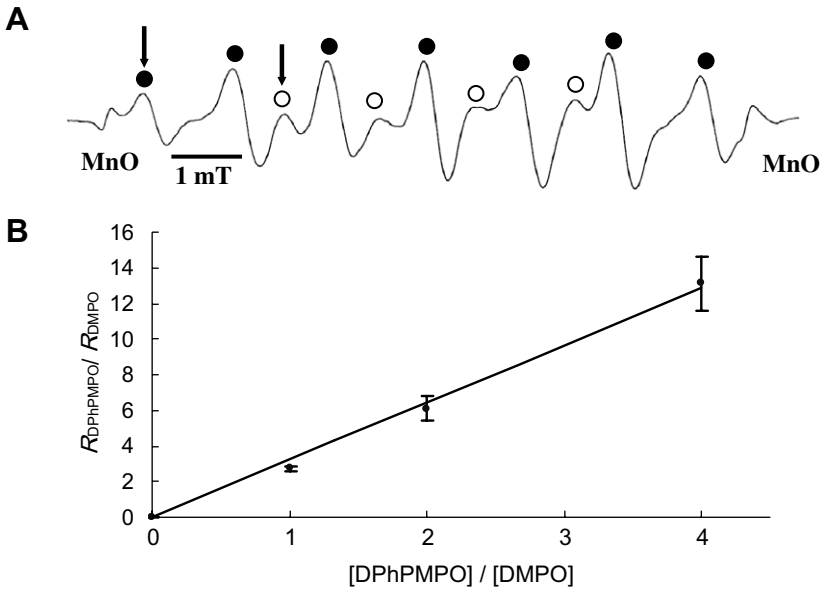

Fig. 2. ESR spectrum obtained using the X/XO system in the presence of 25 mM DPhPMPO and 25 mM DMPO. (A), ●; DPhPMPO-OOH and ○; DMPO-OOH. The ratio of $O_2^{\cdot-}$ spin adduct formation rates for DPhPMPO and DMPO is plotted as a function of the ratio of the initial concentration of the spin traps (B). Data are expressed as means ± SD, *n* = 3.

potential of DPhPMPO for $O_2^{\cdot-}$ was significantly higher than that of DMPO for $O_2^{\cdot-}$. The formation of spin adducts via $O_2^{\cdot-}$ trapping was confirmed in the experiments where SOD was added before PMA, in which no ESR signals were observed (data not shown). By cytochrome *c* reduction assay, a kinetics study for these spin traps was performed using human neutrophils (k_{DMPO} = 13.95 M^{-1} s^{-1} [9] led to $k_{DPhPMPO}$ = 42.4 M^{-1} s^{-1}).

Discussion

ESR spin-trapping is a useful technique for detecting free radicals formed *in vitro* and *in vivo* [21–23]. Because the ESR technique requires a specialized and expensive ESR spectrometer, alternative methods have been commonly used. However, the specificity and reliability of these indirect and non-specific methods are called into question in biological systems [24–26]. Spectroscopic probes such as spin traps, hydroxyamines, spectrophotometric probes, fluorescence probes and luminescence probes are used for free radical detection. Therefore, the high-sensitive and high-specific ability to detect free radicals is the key for developing and characterizing specific probes including new spin traps.

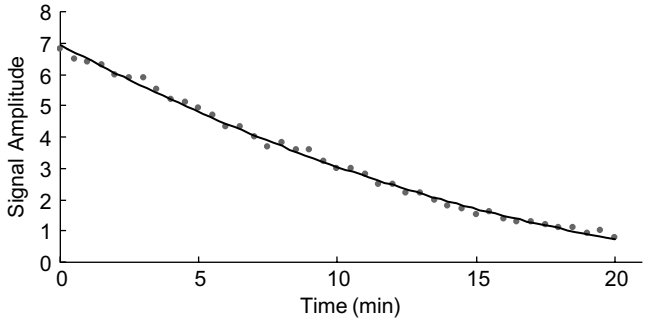

Fig. 3. Experimental and calculated decay of DPhPMPO-OOH generated in the X/XO system. The calculated curve corresponds to first-order decay with a kinetic constant of 0.11 min^{-1}. The curve is shown from one representative experiment out of three.

ESR spin-trapping techniques offer the potential to provide measurements of specific free radicals with high sensitivity and specificity. Although one of the most frequently used spin traps is DMPO, it has several fundamental limitations in terms of half-life and stability. In this study, we compared the abilities of DMPO and our newly developed spin trap DPhPMPO in trapping $O_2^{\cdot-}$ in cell-free and cellular systems. We found that the intensity of DPhPMPO-OOH is more than four times higher than that of DMPO-OOH (Fig. 1) in our cell-free system. The rate constant of DPhPMPO for $O_2^{\cdot-}$ and the half-lives of $O_2^{\cdot-}$ spin adducts of DMPO and DPhPMPO have been obtained. The rate constant of DPhPMPO for $O_2^{\cdot-}$ was 48 M^{-1} s^{-1}, more than three times higher than that of DMPO in the enzymatic $O_2^{\cdot-}$ generation system (Fig. 2). The half-lives of DMPO-OOH and DPhPMPO-OOH were 1.5 ± 0.1 min and 6.2 ± 0.36 min, respectively, indicating that DPhPMPO-OOH was more stable than DMPO-OOH (Fig. 3). These results were nearly consistent with the half-lives of the previously reported DMPO [8] and DPhPMPO [14], and indicated that the ability of DPhPMPO in trapping $O_2^{\cdot-}$ in a cell-free system is superior to that of DMPO.

In our cellular system, the detection potential of DPhPMPO for $O_2^{\cdot-}$ was significantly higher than that of DMPO, as well as in a cell-free system (Fig. 4). Unlike in a cell-free system, the intensity of DPhPMPO-OOH is only about two times higher than that of DMPO-OOH, which may result from its lipophilic property. The partition coefficients (K_p) of DPhPMPO and DMPO in the 1-octanol/water system were 4.3 [14] and 0.1 [27,28], respectively. This

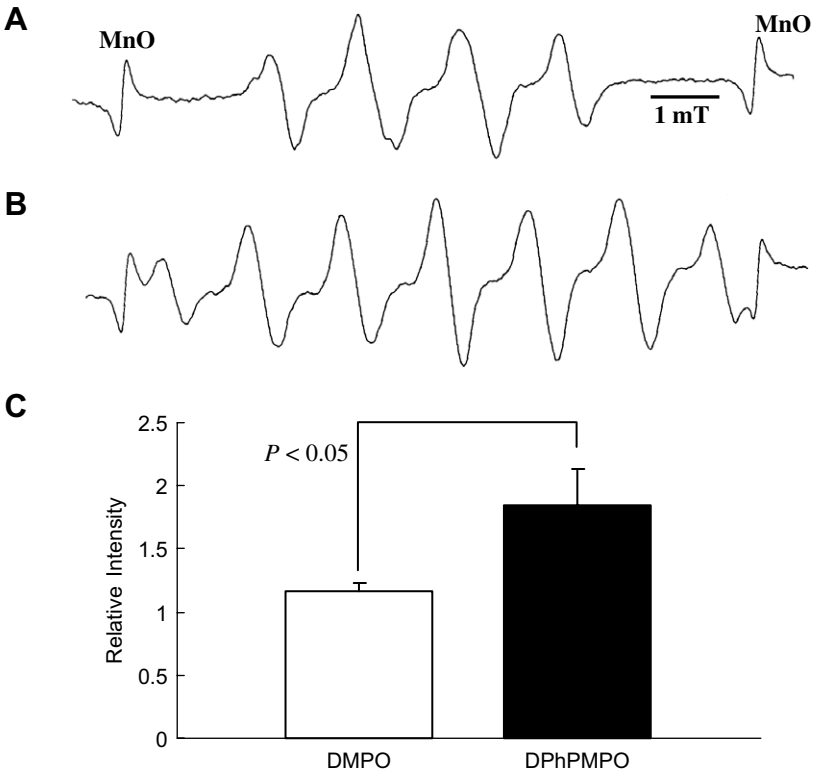

Fig. 4. ESR study in human neutrophils activated by PMA using DPhPMPO or DMPO as a spin traps. Representative ESR spectra of $O_2^{\cdot-}$ spin adducts for DMPO (A) and DPhPMPO (B), and the intensity of $O_2^{\cdot-}$ spin adducts for DMPO and DPhPMPO (C) in human neutrophils activated by PMA. The bar graph reflects the peak-to-peak amplitude of the most intense resonance in the spectrum relative to the amplitude of manganese oxide. Data are expressed as means ± SD, n = 3.

indicates that DPhPMPO is lipophilic and may stay within the cellular membrane.

In fact, the K_p of 2-diethoxy phosphoryl-2-phenethyl-3,4-dihydro-2*H*-pyrrole-1-oxide (DEPPEPO) is 7.6 [27,28], but DEPPEPO was unable to trap cell-generated $O_2^{\cdot-}$ [10]. Consequently, DPhPMPO was able to trap cell-generated $O_2^{\cdot-}$ in this study. K_p is an important parameter for a spin-trapping reagent since high lipophilicity can improve its distribution in the cell membrane. Therefore, DPhPMPO may be a useful spin trap in various systems.

Both DMPO and DPhPMPO were able to trap cell-generated $O_2^{\cdot-}$, although the obtained DMPO signal did not show a pure DMPO-OOH spectrum but rather a mixture of DMPO-OOH and DMPO-OH spectra (Fig. 4). The DPhPMPO signal showed a pure DPhPMPO-OOH spectrum, as well as in the cell-free system. It is known that light, a transition metal, or glutathione peroxidase can convert a $O_2^{\cdot-}$ adduct to a hydroxyl adduct [9–11,20,29]. When SOD was pre-incubated with cells, the observed ESR signal disappeared (data not shown). Thus, the DMPO-OH adduct is generated by the decay of DMPO-OOH, and $O_2^{\cdot-}$ is the primary radical product from neutrophils activated by PMA. This result indicates that one of the main advantages of DPhPMPO is that DPhPMPO-OOH does not readily decay to DPhPMPO-OH.

DPhPMPO is a white solid at room temperature and thus quite easy to store and is much easier to purify and use. Furthermore, DPhPMPO did not degrade in an aqueous solution for several months. During our extensive use of the spin traps, we have not observed any artificial signal of DPhPMPO in the concentration range used. Commercially available DMPO usually needs to be purified before use. In this regard, DPhPMPO has advantages in trapping cell-generated $O_2^{\cdot-}$.

In conclusion, the present study has shown that DPhPMPO reacted more quickly with $O_2^{\cdot-}$ and formed a more stable $O_2^{\cdot-}$ adduct than DMPO. Therefore, DPhPMPO appears to be a better spin trap in cell-free and cellular systems. The approach for quantitative measurement of $O_2^{\cdot-}$ using ESR spin-trapping with the new spin trap DPhPMPO should be applicable to various chemical and biological applications where there has been a great need for quantitative measurements of $O_2^{\cdot-}$ generation.

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