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Singlet Oxygen–mediated Hydroxyl Radical Production in the Presence of Phenols: Whether DMPO–OH Formation Really Indicates Production of OH?

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ABSTRACT

The reaction of singlet oxygen $({}^{1}O_{2})$ generated by ultraviolet-A (UVA)-visible light ($\lambda > 330$ nm) irradiation of air-saturated solutions of hematoporphyrin with phenolic compounds in the presence of a spin trap, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), gave an electron spin resonance (ESR) spectrum characteristic of the DMPO-hydroxyl radical spin adduct (DMPO-'OH). In contrast, the ESR signal of 5,5-dimethyl-2pyrrolidone-N-oxyl, an oxidative product of DMPO, was observed in the absence of phenolic compounds. The ESR signal of DMPO-'OH decreased in the presence of either a 'OH scavenger or a quencher of ${}^{1}O_{2}$ and under anaerobic conditions, whereas it increased depending on the concentration of DMPO. These results indicate both ¹O₂- and DMPOmediated formation of free 'OH during the reaction. When DMPO was replaced with 5-(diethoxyphosphoryl)-5-methyl-1pyrroline-N-oxide (DEPMPO), no DEPMPO adduct of oxygen radical species was obtained. This suggests that ¹O₂, as an oxidizing agent, reacts little with DEPMPO, in which a strong electron-withdrawing phosphoryl group increases the oxidation potential of DEPMPO compared with DMPO. A linear correlation between the amounts of DMPO-'OH generated and the oxidation potentials of phenolic compounds was observed, suggesting that the electron-donating properties of phenolic compounds contribute to the appearance of 'OH. These observations indicate that ${}^{1}O_{2}$ reacts first with DMPO, and the resulting DMPO $-^{1}O_{2}$ intermediate is immediately decomposed/reduced to give 'OH. Phenolic compounds would participate in this reaction as electron donors but would not contribute to the direct conversion of ¹O₂ to [•]OH. Furthermore, DEPMPO did not cause the spin-trapping agentmediated generation of 'OH like DMPO did.

INTRODUCTION

Reactive oxygen species (ROS) such as superoxide $(O_2^{\bullet-})$, the hydroxyl radical (•OH) and singlet oxygen (${}^{1}O_{2}$) have been implicated both in the aging process and in degenerative disease (1–3). $O_2^{\bullet-}$ and •OH are oxygen radicals produced by electron donation to molecular oxygen, whereas ${}^{1}O_2$ is generated from molecular oxygen by energy transfer. As well as •OH, the most reactive oxygen radical, ${}^{1}O_2$ oxidizes many biological substances such as amino acids, polyunsaturated fatty acids and nucleosides (4,5), thereby causing cell death and mutations (6).

The interaction of ${}^{1}O_{2}$ with some compounds may generally lead to the generation of molecular oxygen by energy transfer reactions or to other ROS including O2^{•-} by electron transfer reactions (or both) (7). Concerning the latter case, Saito *et al.* (8) reported that organic compounds with oxidation potentials of less than 0.5 V (*vs* SCE) were capable of undergoing single-electron transfer to ${}^{1}O_{2}$ to generate O2^{•-} preferentially in aqueous solvents. On the other hand, the possibility of direct conversion of ${}^{1}O_{2}$ to •OH by biological reductants such as reduced glutathione and reduced nicotinamide adenine dinucleotide phosphate (NADPH) has been suggested (9,10).

Skin is a defensive barrier and is constantly exposed to certain kinds of oxidative stress such as ultraviolet and ionizing irradiation (11). ${}^{1}O_{2}$ is produced through the interaction of the ultraviolet-A component (UVA) of sunlight with endogenous photosensitizers such as porphyrins and flavins in the skin (6,12). Because phenolic compounds are widely used in the production of pharmaceutical as well as cosmetic and food-flavoring products, the skin is inevitably exposed to phenolic compounds (13). Therefore, it is considered that a phenolic compound, which is as potent an electron donor as reduced glutathione or NADPH, may also cause the conversion of ${}^{1}O_{2}$ to another ROS. Then, we attempted to elucidate whether oxygen radicals are produced from ${}^{1}O_{2}$ in the presence of phenolic compounds.

In this study, ${}^{1}O_{2}$ was generated by irradiation of hematoporphyrin (HP), a model compound for endogenous porphyrins in the skin (14), with UVA-visible light ($\lambda > 330$ nm), and the assignment of oxygen radicals was performed by electron spin

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Abbreviations: DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; DMPOX, 5,5-dimethyl-2-pyrrolidone-N-oxyl; ESR, electron spin resonance; HP, hematoporphyrin; NADPH, reduced nicotinamide adenine dinucleotide phosphate; ¹O₂, singlet oxygen; O₂^{•-}, superoxide; [•]OH, hydroxyl radical; ROS, reactive oxygen species; TEMPD, 2,2,6,6-tetramethyl-4-piperidone; TEMPON, 2,2,6,6-tetramethyl-4-piperidone-1-oxyl; UVA, ultraviolet-A.

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Figure 1. Chemical structures of the phenolic compounds used.

resonance (ESR) using as a spin trap, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). DMPO is useful for identifying oxygen-centered radicals because the resultant spin adducts have characteristic ESR parameters and can be readily distinguished from other spin adducts. The chemical structures of nine phenolic compounds examined in this study are shown in Fig. 1. We observed the production of the DMPO adduct of °OH (DMPO–°OH) derived from ${}^{1}O_{2}$ in the presence of phenolic compounds and examined whether the presence of the adduct really reflects the generation of free °OH during the reaction of ${}^{1}O_{2}$ with phenolic compounds.

MATERIALS AND METHODS

Materials. Trolox C, isoeugenol, *p*-hydroxybenzoic acid, 2,2,6,6-tetramethyl-4-piperidone (TEMPD) and 2,2,6,6-tetramethyl-4-piperidone-1oxyl (TEMPON) were purchased from Aldrich Chemical Co. (Milwaukee, WI). *p*-Eugenol, *p*-methoxyphenol, phenol, 2,6-dimethoxyphenol and guaiacol were obtained from Wako Pure Chemical Co. (Osaka, Japan). HP and dopamine were purchased from Nakarai Chemical Co. (Kyoto, Japan). DMPO was provided by Labotec (Tokyo, Japan) and used without further purification. 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) was purchased from Oxis International Inc. (Portland, OR) and also used without further purification. Chelex 100 resin (sodium form) was obtained from Bio-Rad Co. (Hercules, CA).

Generation and reaction with phenolic compounds of ${}^{1}O_{2}$. ${}^{1}O_{2}$ was generated by irradiating HP with UVA–visible light as described previously (15) and was detected as an oxidative product, TEMPON, of TEMPD with ${}^{1}O_{2}$ (16,17). Furthermore, the occurrence of ${}^{1}O_{2}$ was confirmed from the inhibition of TEMPON formation by histidine or sodium azide (NaN₃), known ${}^{1}O_{2}$ quenchers. UVA–visible light was irradiated through a UV-33 filter (cut-off: 330 nm, Toshiba Glass Co., Tokyo, Japan) using Supercure-203S with a 200 W Mercury–Xenon lamp (Radical Research Inc., Tokyo, Japan) connected to the ESR cavity by a quartz fiber. The dose was 142 mW/cm² at 365 nm on the surface of the flat cell (JEOL, Tokyo, Japan). One-hundred microliters of 100 μ M HP, various concentrations of phenolic compounds and 100 μ L of 400 mM DMPO or DEPMPO were added to 0.1 *M* phosphate buffer (total volume, 400 μ L). After a quick stir, the reaction mixture was transferred into a flat quartz cell. ESR spectra were measured immediately after the appropriate irradiation time.

Measurements of oxidation potentials. To characterize the chemical structure of phenolic compounds, we determined oxidation potentials in phosphate buffer (pH 7.4) using a cyclic voltammetric method. Using an ALS 630A Electrochemical Analyzer (BAS Co., Tokyo, Japan), cyclic voltammetric measurements were made using a three-electrode system consisting of a glass carbon electrode, a platinum counter electrode and an Ag–AgCl electrode as a reference. To remove dissolved oxygen, nitrogen gas was passed through the sample solution for 15 min. Cyclic voltammetry indicated that the oxidation of phenols was not reversible. Therefore, the experimental half-peak oxidation potentials were used as the oxidation potential E_{pa} and further calculated with the equation E^0 (NHE) = E_{pa} + 0.212 (V).

ESR measurements. ESR measurements were made on a JEOL FE-2X spectrometer (X-band) with 100 kHz field modulation. ESR spectra were recorded at room temperature.

Statistical analysis. Linear regression analysis was performed using Statview-4.5J (Abacus Concepts, Berkeley, CA) and used to determine the relation between the signal intensities of DMPO adducts and the oxidation potential of phenolic compounds.

RESULTS

¹O₂-mediated DMPO–[•]OH formation in the presence of phenolic compounds

A four-line ESR signal was observed 2 s after UVA–visible light ($\lambda > 330$ nm) irradiation of an air-saturated solution containing 100 mM DMPO and 25 μ M HP at pH 7.4 in the presence of 100 μ M dopamine (Fig. 2b) but not in the absence of dopamine (Fig. 2a). The hyperfine splitting constants ($a^{N} = a^{H} = 14.9$ G) of this signal corresponded to those of DMPO–•OH (18). No signal from DMPO–•OH was seen without HP, suggesting that DMPO–•OH is derived from the photochemistry dependent on the sensitizer (Fig. 2c). Furthermore, DMPO–•OH was vastly diminished either under anaerobic conditions (Fig. 2d) or with a $^{1}O_{2}$ quencher, NaN₃ (Fig. 2e), indicating that its formation is mediated by $^{1}O_{2}$. No signal for the DMPO adduct of the azide radical (•N₃) was observed.

To elucidate whether the DMPO-•OH adduct was derived either from the decomposition of the DMPO adduct of $O_2^{\bullet-}$ (DMPO- $O_2^{\bullet-}$) or from the reaction of free •OH with DMPO, the photoreaction was performed in the presence of •OH scavengers such as ethanol (5%) and sodium formate (0.25 *M*). The irradiation of the respective solutions gave a new six-line ESR signal corresponding to the DMPO adduct of the α -hydroxyethyl radical (•CH(CH₃)OH) ($a^N = 15.8 \text{ G}, a^H = 22.8 \text{ G}$) (18) (Fig. 2f) or to that of the carbon dioxide anion radical (•COO⁻) ($a^N = 15.6 \text{ G}, a^H = 18.7 \text{ G}$) (18) (Fig. 2g) with a reduction in the signal intensity of the DMPO-•OH adduct, indicating the 1O_2 -mediated formation of free •OH during the photoreaction of HP in the presence of dopamine. Catalase had little effect on the signal intensity of DMPO-•OH (data not shown), indicating that the formation of •OH is not mediated by hydrogen peroxide.

The effect of a substituent group of phenol on DMPO-*OH formation

The time course of the signal intensity of the DMPO-•OH adduct was examined in the presence of various concentrations of dopamine (Fig. 3). The signal intensity of DMPO-•OH increased upon irradiation, reached a maximal level at around 2 s and decreased thereafter. This suggests the production of DMPO-•OH adduct at an initial stage and subsequent decomposition. Because a similar decline in DMPO-•OH signal was observed in most phenolic compounds after irradiation for longer than 2 s, the ESR



Figure 2. ESR spectra due to DMPO adduct detected in a reaction mixture containing NaN₃, ethanol or HCOONa. (A) Complete system containing 25 μ M HP and 100 mM DMPO in 100 mM phosphate buffer, pH 7.4. (B) as in (A) but with addition of 100 μ M dopamine. (C) as in (B) but without HP. (D) as in (B) except that sample was purged with N₂ gas for 15 min before irradiation. (E) as in (B) but with addition of 10 mM NaN₃. (F) as in (B) but with 5% ethanol. (G) as in (B) but with 1 M HCOONa. (C) DMPO adduct of $^{\circ}$ OH; \diamond : DMPO adduct of $^{\circ}$ CH(OH)CH₃; Δ : DMPO adduct of $^{\circ}$ CO₂ $^{-\circ}$. Conditions: microwave power, 10 mW; modulation amplitude, 0.79 G; scan range, 100 G; amplitude, 790 (1000 in the case of A); time constant, 0.03 s; scan time, 2 min.

signal intensities of the DMPO-•OH adduct in the presence of various phenolic compounds were compared from 2 s after irradiation.

The relative ESR signal intensities of DMPO-OH were plotted against the concentrations of phenolic compounds including dopamine at a constant concentration of DMPO as shown in Fig. 4. Phenolic compounds, except for phenol and p-hydroxybenzoic acid, increased the amount of DMPO-OH with an increase in concentration at relatively low levels (less than 250 µM). As shown in Fig. 5, there existed a linear relationship (correlation coefficient: r = 0.918) between the signal intensity of DMPO-•OH in 100 µM phenolic compounds and oxidation potential. A similar relationship was obtained at concentrations below 250 μ M. This suggests that the electron-donating properties of phenolic compounds contribute to the formation of •OH. A further increase in the concentration of phenolic compounds except for Trolox C and isoeugenol led to a plateau in the signal intensity of DMPO-OH, whereas an abrupt decrease in the signal intensity of DMPO-OH was obtained at a relatively high concentration of Trolox C and isoeugenol (Fig. 4).

Involvement of DMPO in the formation of 'OH

When the ESR signal intensity of the DMPO– $^{\circ}$ OH adduct was measured in the presence of 100 μ M dopamine, it was found to increase with the concentration of DMPO, and the increase did not



Figure 3. Time course of DMPO-•OH adduct formation in the presence of various concentrations of dopamine. \Box : 20 μ *M*, \diamond : 100 μ *M*, \odot : 250 μ *M*, Δ : 500 μ *M* and ∇ : 1 m*M*. Data are the average of three experiments.

reach a point of saturation until at least 100 m*M* DMPO (Fig. 6). In contrast, the increase in the signal intensity of the DMPO–•OH adduct produced from the reaction of DMPO with •OH, which was generated by the Fenton reaction, was saturated at 50 m*M* DMPO. This suggests the participation of DMPO in the ${}^{1}O_{2}$ -mediated formation of free •OH.

To ascertain the role of DMPO in the conversion of ${}^{1}O_{2}$ to ${}^{\circ}OH$, a spin-trapping experiment was undertaken using DEPMPO instead of DMPO. The irradiation of a solution containing 25 μ M HP and 100 mM DEPMPO with UVA-visible light did not produce any spin adduct irrespective of the presence or absence of 100 μ M dopamine (Fig. 7a,b), although the reaction rate constant for DEPMPO with ${}^{\circ}OH$ (7.1 \times 10⁹ M^{-1} s⁻¹) is slightly higher than that for DMPO (3.4 \times 10⁹ M^{-1} s⁻¹) (19). When DMPO was added to the reaction mixture described above (Fig. 7c), however, an eight-line ESR signal appeared in addition to the four-line signal due to DMPO- ${}^{\circ}OH$. The hyperfine splitting constants (a^P = 46.8 G, a^N = 13.8 G, a^H = 13.7 G) of this eight-line ESR signal corresponded to those of the DEPMPO adduct of hydroxyl radical (DEPMPO- ${}^{\circ}OH$) (19). These results strongly indicate that the formation of ${}^{\circ}OH$ is dependent on DMPO.

The formation of 5,5-dimethyl-2-pyrrolidone-*N*-oxyl in the absence of phenolic compounds

The oxidative product of DMPO, 5,5-dimethyl-2-pyrrolidone-*N*-oxyl (DMPOX) ($a^N = 7.2$ G, $a(2)^H = 4.1$ G) (20), was obtained 20 s after UVA irradiation of an air-saturated solution of 25 μ *M* HP and 20 m*M* DMPO in the absence of phenolic compounds (Fig. 8a). No signal for DMPO–•OH was found, although it has been reported that DMPO reacts with ${}^{1}O_2$ generated by the reaction of excited photosensitizers with visible light to yield DMPO–•OH (21–25). The signal intensity of DMPOX increased with time, reached a maximum and subsequently decreased as shown in Fig. 8b. The increase in DMPO concentration accelerated the initial rate of production of DMPOX but hastened the reduction of DMPOX signal. In contrast, no DMPOX signal was observed



Figure 4. Effect of varying the concentration of phenolic compounds on the ESR signal intensity of DMPO adduct of **•**OH radical in the presence of 100 m*M* DMPO. \Box : Trolox C, \diamond : isoeugenol, \bigcirc : *p*-eugenol, Δ : *p*-methoxyphenol, ∇ : guaiacol, \blacksquare : dopamine, \blacklozenge : 2,6-dimethoxyphenol, \blacklozenge : phenol and \blacktriangle : p-hydroxybenzoic acid. Data are the average of two or three experiments.

in the presence of phenolic compounds at any DMPO concentration (data not shown).

DISCUSSION

The exposure of an air-saturated solution containing dopamine, DMPO and HP to UVA-visible light ($\lambda > 330$ nm) led to the formation of DMPO-OH adduct. No signal for DMPO-OH was obtained without HP, suggesting that DMPO-OH is from the photosensitizer-dependent reaction of DMPO and not from the direct photochemical processes including the photoionization of DMPO. A competitive scavenging experiment using both an ${}^{1}O_{2}$ quencher, NaN₃, and OH scavengers, such as ethanol and sodium formate, clearly indicated that the DMPO trapped free OH, which was formed through a ${}^{1}O_{2}$ -mediated pathway in the presence of dopamine.

Although the displacement of DMPO with DEPMPO, in which one methyl group at position 5 of DMPO is substituted with a diethoxyphosphoryl group, did not give any ESR signal for its •OH adduct, DEPMPO-•OH, a remarkable signal for the DEPMPO-OH adduct was observed on addition of DMPO to the reaction mixture, strongly suggesting that the formation of 'OH was mediated by DMPO. It has been reported that DMPO itself reacted with ${}^{1}O_{2}$ (k = 1.8 × 10⁷ \dot{M}^{-1} s⁻¹) (22) to yield DMPO-OH and free OH via the decomposition of the reaction intermediate of DMPO with ¹O₂ (21-25). Bilski et al. (25) reported that the reaction is initiated by the electrophilic reaction of ${}^{1}O_{2}$ at position 2 of DMPO. The existence of a strong electronwithdrawing phosphoryl group in DEPMPO may reduce the electron density of the C-N bond in DEPMPO compared with that in DMPO. This suggestion is supported by the report that the oxidation potential of a nonphosphorylated compound, *a*-phenyl-N*tert*-butylnitrone (Ep[a] = 1.47 V vs SCE in acetonitrile) was lower

[Phenolic compounds] = $100 \,\mu$ M



Figure 5. Correlation between signal intensities of DMPO–[•]OH adducts and oxidation potential of phenolic compounds at a concentration of 100 μ M. Numbers correspond to those in Fig. 1.

than that of the phosphorylated form (Ep[a] = 1.60 V vs SCE) (26). Then, ${}^{1}O_{2}$ may hardly react with DEPMPO. This may explain why no DEPMPO-OH was found in the absence of DMPO. The reaction of DMPO with ${}^{1}O_{2}$ should form a nitronium-like compound with C-hydroperoxide (-CH(OO⁻)-N⁺(=O)-) (intermediate I) as shown in Scheme 1.

Phenolic compounds affected the formation of the DMPO-•OH adduct, depending on their substituent group. Because the concentration of DMPO is larger than that of phenolic compounds, ¹O₂ reacts preferentially with DMPO to produce intermediate (I). Therefore, phenolic compounds may react either with photoactivated HP (triplet state) or with intermediate (I) as an electron donor. The reduction of photoactivated HP by phenolic compounds may not contribute to the formation of DMPO-OH because it should decrease the generation of DMPO-OH by suppressing ¹O₂ production. Phenolic compounds, except for phenol and phydroxybenzoic acid, increased the intensity of DMPO-OH in a dose-dependent manner at relatively low concentrations of phenolic compounds. In addition, a linear relationship between the signal intensity of DMPO-OH in the presence of phenolic compounds and the oxidation potential of the corresponding compounds was obtained. Thus, these results suggest that phenolic compounds act as electron donors, probably decomposing intermediate (I) leading to the ${}^{1}O_{2}$ -mediated formation of ${}^{\bullet}OH$ as shown in Scheme 1. No phenoxyl radical was observed in the presence of MgCl₂, a stabilizer of the semiquinone radical, probably because of its very short life span (data not shown). The rapid reaction of ${}^{1}O_{2}$ with a wide range of phenolic compounds was reported to produce endoperoxides and then hydroperoxides, and the decomposition of the hydroperoxides by UV may produce 'OH because it was reported that UV irradiation of phthalimide hydroperoxide produces 'OH (27). In the present case, however, the contribution of this reaction to the formation of •OH would be minor for the following reasons: (1) no •OH was observed without DMPO in the spin-trapping with DEPMPO, suggesting that the formation of 'OH is mediated by DMPO and (2) a preferential reaction of ${}^{1}O_{2}$ with DMPO is presumed because



Figure 6. Dependence of DMPO-[•]OH signal amplitude obtained from both photochemical and Fenton reactions on DMPO concentrations. DMPO-[•]OH was obtained by UVA-visible light ($\lambda > 330$ nm) irradiation of an air-saturated solution containing 25 μ M HP and 100 μ M dopamine (\bigcirc) and from a reaction mixture containing 12.5 μ M FeSO₄, 100 μ M DTPA and 250 μ M H₂O₂ (\diamondsuit). Data are the average of two or three experiments.

the concentration of DMPO is much higher than that of phenolic compounds, as discussed above. The decrease of DMPO-•OH signal in the presence of excess Trolox C and isoeugenol may be due to their strong •OH scavenging activity or abnormal reducing ability (or both). Trolox C and isoeugenol markedly enhanced the spin elimination of a nitroxyl radical TEMPON during the HP-photoreaction but other phenolic compounds did not (data not shown).

In the absence of phenolic compounds, DMPOX instead of DMPO–•OH was observed. Bilski *et al.* (25) described the formation of DMPOX in a photodynamic reaction of micellar rose bengal in the presence of DMPO at pH 2 and proposed that intermediate (I) oxidized DMPO–•OH to yield DMPOX. DMPOX is usually formed during the oxidation of DMPO by strong oxidizers (20,28–30). Thus, it is likely that DMPOX is produced



Figure 7. ESR spectra due to DMPO adduct detected in a reaction mixture containing DEPMPO and dopamine. (A) Complete system containing 25 μ M HP and 100 mM DEPMPO in 100 mM phosphate buffer, pH 7.4. (B) as in (A) but with addition of 100 μ M dopamine. (C) as in (B) but with 20 mM DMPO. \bigcirc : DMPO adduct of °OH; Δ : DEPMPO adduct of °OH. Conditions: microwave power, 10 mW; modulation amplitude, 0.79 G; scan range, 100 G; amplitude, 790; time constant, 0.03 s; scan time, 2 min.



Figure 8. (a) Spectrum of DMPOX and (b) time course of signal intensity of DMPOX in the presence of various concentrations of DMPO. (A) Spectrum measured 20 s after UVA–visible light irradiation of a reaction mixture containing 25 μ M HP and 20 mM DMPO. Conditions: microwave power, 10 mW; modulation amplitude, 0.79 G; scan range, 100 G; amplitude, 790; time constant, 0.03 s; scan time, 2 min. (B) [DMPO] = \Box : 10 mM; \diamond : 20 mM; \bigcirc : 40 mM and Δ : 100 mM. Data are the average of two experiments.

from the oxidation of DMPO by intermediate (I). The decomposition of intermediate (I) by phenolic compounds, if it occurs, may inhibit the formation of DMPOX and enhance the formation of DMPO-•OH (Scheme 1). This hypothesis is



Scheme 1. Sequence for the formation of DMPO– $^{\bullet}$ OH or DMPOX by the reaction of ${}^{1}O_{2}$ with DMPO in the presence or absence of phenolic compounds.

supported by the disappearance of DMPOX and the appearance of DMPO-•OH in the presence of phenolic compounds.

Prolonged irradiation caused the signal intensity of DMPO-•OH to decrease. The decrease may be explained as follows. The excess reaction caused by the long irradiation produced a large amount of DMPO-•OH accompanied by the consumption of oxygen and the subsequent formation of HP anion radical, which was generated *via* a Type-I mechanism under anaerobic conditions, resulting in the spin elimination of DMPO-•OH by HP anion radical (21). A similar reduction was observed in the signal for DMPOX after prolonged irradiation. The presence of phenolic compounds prevented a weakening of the DMPO-•OH signal (Fig. 4). This suggests that inhibition of the loss of DMPO-•OH signal is another possible mechanism for the appearance of DMPO-•OH only in the presence of phenolic compounds.

In conclusion, the ${}^{1}O_{2}$ -mediated formation of •OH in the presence of phenolic compounds was obviously initiated by the reaction of ${}^{1}O_{2}$ with DMPO used as a spin trap. Phenolic compounds would participate in the production of •OH as electron donors but not in the direct conversion of ${}^{1}O_{2}$ to •OH. Furthermore, DEPMPO did not cause the generation of •OH.

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REFERENCES

- 1. Ames, B. N. (1983) Dietary carcinogens and anticarcinogens. *Science* 221, 1256–1264.
- Cerutti, P. A. (1985) Prooxidant states and tumor promotion. *Science* 227, 375–381.
- Halliwell, B. and J. M. C. Gutteridge (1999) In *Free Radicals in Biology and Medicine*, 3rd ed., Oxford University Press, New York, NY.
- Decuyper-Debergh, D., J. Piette, C. Laurent and A. van de Vorst (1989) Cytotoxic and genotoxic effects of extracellular generated singlet oxygen in human lymphocytes in vitro. *Mutat. Res.* 225, 11–14.
- Ravanat, J.-L., P. Di Mascio, G. R. Martuez, M. H. G. Medeiros and J. Cadet (2000) Singlet oxygen induces oxidation of cellular DNA. *J. Biol. Chem.* 275, 40601–40604.
- Ryter, S. W. and R. M. Tyrrell (1998) Singlet molecular oxygen (¹O₂): a possible effector of eukaryotic gene expression. *Free Radic. Biol. Med.* 24, 1520–1534.
- Foote, C. S. (1976) "Photosensitized oxidation and singlet oxygen: consequences in biological systems. In *Free Radicals in Biology*, Vol. II (Edited by W. A. Pryor), pp. 85–133. Academic Press, New York.
- Saito, I., T. Matsuura and K. Inoue (1983) Formation of superoxide ion via one-electron transfer from electron donors to singlet oxygen. J. Am. Chem. Soc. 105, 3200–3206.
- Buettner, G. R. (1985) Thiyl free radical production with hematoporphyrin derivative, cysteine and light: a spin-trapping study. *FEBS Lett.* 177, 295–299.
- Takeshita, K., C. A. Olea-Azar, M. Mizuno and T. Ozawa (2000) Singlet oxygen-dependent hydroxyl radical formation during uroporphyrin-mediated photosensitization in the presence of NADPH. *Antiox. Redox Signal.* 2, 355–362.
- Fuchs, J., N. Groth, T. Herrling and G. Zimmer (1997) Electron paramagnetic resonance studies on nitroxide radical 2,2,5,5-tetramethyl-4-piperidin-1-oxyl (TEMPO) redox reactions in human skin. *Free Radic. Biol. Med.* 22, 967–976.

- Ito, K. and S. Kawanishi (1997) Site-specific DNA damage induced by UVA radiation in the presence of endogenous photosensitizer. *Biol. Chem.* 378, 1307–1312.
- Shvedova, A. A., C. Kommineni, B. A. Jeffries, V. Castranova, Y. Y. Tyrina, V. A. Tyurin, E. A. Serbinova, J. P. Fabisiak and V. E. Kagan (2000) Redox cycling of phenol induces oxidative stress in human epidermal keratinocytes. *J. Investig. Dermatol.* **114**, 354–364.
- Ryu, A., E. Naru, K. Arakane, T. Masunaga, K. Shinmoto, T. Nagano, M. Hirobe and S. Mashiko (1997) Cross-linking of collagen by singlet oxygen generated with UV-A, *Chem. Pharm. Bull.* 45, 1243–1247.
- Osada, M., Y. Ogura, H. Yasui and H. Sakurai (1999) Involvement of singlet oxygen in cytochrome P450-dependent substrate oxidations. *Biochem. Biophys. Res. Commun.* 263, 392–397.
- Moan, J. and E. Wold (1979) Detection of singlet oxygen production by ESR. *Nature* 279, 450–451.
- Lion, Y., M. Delmelle and A. van de Vorst (1976) New method of detecting singlet oxygen production. *Nature* 263, 442–443.
- Buettner, G. R. (1987) Spin trapping: ESR parameters of spin adducts. Free Radic. Biol. Med. 3, 259–303.
- Frejaville, C., H. Karoui, B. Tuccio, F. Le Moigne, M. Culcasi, S. Pietri, R. Lauricella and P. Torde (1995) 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide: a new efficient phosphorylated nitrone for the in vitro and in vivo spin trapping of oxygen-centered radicals. *J. Med. Chem.* 38, 258–265.
- Ozawa, T., Y. Miura and J. Ueda (1996) Oxidation of spin-traps by cholorine dioxide (ClO2) radical in aqueous solutions: first ESR evidence of formation of new nitroxide radicals. *Free Radic. Biol. Med.* 20, 837–841.
- Zhang, S.-P., J. Xie, J.-P. Zhang, J.-Q. Zhao and L.-J. Jiang (1999) Electron spin resonance studies on photosensitized formation of hydroxyl radical by c-phycocyanin from Spirulina plantensis. *Biochim. Biophys. Acta* 1426, 205–211.
- Harbour, J. R., S. L. Issler and M. L. Hair (1980) Singlet oxygen and spin trapping with nitrones. J. Am. Chem. Soc. 102, 7778–7779.
- 23. Hoebeke, M., H. J. Schuitmaker, L. E. Jannink, T. M. A. R. Dubbelman, A. Jakobs and A. van de Vorst (1997) Electron spin resonance of the generation of superoxide anion, hydroxyl radical and singlet oxygen during the photohemolysis of human erythrocytes with bacteriochlorin a. *Photochem. Photobiol.* **66**, 502–508.
- 24. Feix, J. B. and B. Kalyanaraman (1991) Production of singlet oxygenderived hydroxyl radical adducts during merocyanine-540-mediated photosensitization: analysis by ESR-spin trapping and HPLC with electrochemical detection. *Arch. Biochem. Biophys.* 291, 43–51.
- Bilski, P., K. Reszka, M. Bilska and C. F. Chignell (1996) Oxidation of the spin trap 5,5-dimethyl-1-pyrroline N-oxide by singlet oxygen in aqueous solution. J. Am. Chem. Soc. 118, 1330–1338.
- Tuccio, B., P. Bianco, J. C. Bouteiller and P. Tordo (1999) Electrochemical characterisation of β-phosphorylated nitrone spin traps. *Electrochim. Acta* 44, 4631–4634.
- Saito, I., M. Takayama, T. Matsuura, S. Matsugo and S. Kawanishi (1990) Phthalimide hydroperoxides as efficient photochemical hydroxy radical generators. A novel DNA-cleaving agent. J. Am. Chem. Soc. 112, 883–884.
- Rosen, G. M. and E. J. Rauckman (1980) Spin trapping of the primary radical involved in the activation of the carcinogen N-hydroxy-2acetylaminofluorene by cumene hydroperoxide-hematin. *Mol. Pharmacol.* 17, 233–238.
- Mao, G. D., P. D. Thomas and M. Poznansky (1994) Oxidation of spin trap 5,5-dimethyl-1-pyrroline-1-oxide in an electron paramagnetic resonance study of the reaction of methemoglobin with hydrogen peroxide. *Free Radic. Biol. Med.* 16, 493–500.
- Gunther, M. R., R. A. Tschirret-Guth, H. E. Witkowska, Y. C. Fann, D. P. Barr, P. R. Ortiz de Montellano and R. P. Mason (1998) Sitespecific spin trapping of tyrosine radicals in the oxidation of metmyoglobin by hydrogen peroxide. *Biochem. J.* 330, 1293–1299.