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Hydroxyl Radical Generation Caused by the Reaction of Singlet Oxygen with a Spin Trap, DMPO, Increases Significantly in the Presence of Biological Reductants

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Photosensitizers newly developed for photodynamic therapy of cancer need to be assessed using accurate methods of measuring reactive oxygen species (ROS). Little is known about the characteristics of the reaction of singlet oxygen ($^1O_2$) with spin traps, although this knowledge is necessary in electron spin resonance (ESR)/spin trapping. In the present study, we examined the effect of various reductants usually present in biological samples on the reaction of $^1O_2$ with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). The ESR signal of the hydroxyl radical (·OH) adduct of DMPO (DMPO-OH) resulting from $^1O_2$-dependent generation of ·OH strengthened remarkably in the presence of reduced glutathione (GSH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ascorbic acid, NADPH, etc. A similar increase was observed in the photosensitization of uroporphyrin (UP), rose bengal (RB) or methylene blue (MB). Use of 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO) as a spin trap significantly lessened the production of its ·OH adduct (DEPMPO-OH) in the presence of the reductants. The addition of DMPO to the DEPMPO-spin trapping system remarkably increased the signal intensity of DEPMPO-OH. DMPO-mediated generation of ·OH was also confirmed utilizing the hydroxylation of salicylic acid (SA). These results suggest that biological reductants enhance the ESR signal of DMPO-OH produced by DMPO-mediated generation of ·OH from $^1O_2$, and that spin trap-mediated ·OH generation hardly occurs with DEPMPO.

Keywords: DMPO; DEPMPO; Salicylic acid; Singlet oxygen; Hydroxyl radical; Antioxidants

Abbreviations: DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide; ROS, reactive oxygen species; UP, uroporphyrin; ESR, electron spin resonance; SA, salicylic acid; DHBA, dihydroxybenzoic acid; RB, rose bengal; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl; MB, methylene blue; ·OH, hydroxyl radical; $O_2^{\cdot-}$, superoxide anion radical; $^1O_2$, singlet oxygen

INTRODUCTION

Reactive oxygen species (ROS) such as hydroxyl radical (·OH), superoxide anion radical ($O_2^{\cdot-}$) and singlet oxygen ($^1O_2$) are capable of causing damage to various cellular constituents such as DNA, proteins and lipids, leading to carcinogenesis, aging and many other diseases.[1–12] In contrast, ROS are actively utilized in photodynamic and radiation therapy for malignant carcinoma. It is known that the reactivities and toxic effects of ROS differ. Accurate measurements of ROS are essential to clarify how ROS are generated and its relation to tissue damage. They are also necessary for the assessment of therapeutic methods utilizing ROS; e.g. the development of new photosensitizers and the selection of effective radiation.

5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) is widely used as a spin trap to identify many radicals including oxygen radicals, because ESR spectra of various radical adducts of DMPO are well characterized.[13] The reaction of DMPO with $^1O_2$, non-radical ROS, to yield ·OH adduct of DMPO (DMPO-OH) was first demonstrated by Harbour

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et al.[14] Feix and Kalyanaraman[15] suggested that DMPO reacted with $^1O_2$ generated by a photosensitizing reaction of merocyanine 540-treated liposomes to yield free ·OH which forms DMPO-OH through a reaction with unreacted DMPO. Bilski *et al.*[16] also speculated that the addition of $^1O_2$ to $>C=N^+(O^-)$- bond of DMPO led to the generation of free ·OH in the photosensitization of micellar rose bengal (RB). Formation of DMPO-OH via oxidation of DMPO by $^1O_2$ was also suggested in the photosensitization of titanium dioxide.[17] These reports demonstrated the possibility of the artificial formation of ·OH when DMPO is used as a spin trap in photosensitizing reactions. In order to prevent misunderstandings in the identification of ROS, therefore, it is necessary to have a detailed knowledge of the reaction of spin traps with $^1O_2$.

We have observed previously that the ESR signal intensity of DMPO-OH increased remarkably during the photosensitizing reaction of uroporphyrin (UP) in the presence of NADPH.[18] Only a faint signal was detected on the addition of either a ·OH scavenger or $^1O_2$ quencher, suggesting that the formation of DMPO-OH results from the $^1O_2$-dependent generation of ·OH. The $^1O_2$-dependent generation of ·OH was also observed to be enhanced during the photosensitization of hematoporphyrin in the presence of phenolic compounds with low redox potential, indicating that the reducing activity of coexisting compounds is involved in the enhancement.[19] Various reductants including reduced glutathione (GSH), ascorbic acid and tocopherols are present in living organs as well as NADPH. If the enhancement of the $^1O_2$-dependent generation of ·OH is attributable to the reducing activity of coexisting compounds, a similar reaction may occur in biological samples containing these reductants.

In the present study, we demonstrated the increased production of $^1O_2$-derived ·OH in the presence of biological reductants (GSH, ascorbic acid and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), a water-soluble homologue of tocopherol, etc.) as previously observed with NADPH, and then examined whether this production is based on an artificial reaction mediated by DMPO or not, using spin trapping of ·OH with 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO) and salicylic acid (SA).

MATERIALS AND METHODS

Materials

UP I dihydrochloride was obtained from Frontier Scientific, Inc. (Logan, UT, USA). RB, methylene blue (MB), reduced GSH, ascorbic acid, 5-hydroxy-L-tryptophan, 2,3-dihydroxybenzoic acid (2,3-DHBA), 2,5-dihydroxybenzoic acid (2,5-DHBA) and catechol were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). SA and dihydrolipoic acid were obtained from Sigma Inc. (St Louis, MO, USA). DMPO was purchased from Labotec (Tokyo, Japan). DEPMPO was obtained from Oxis International (Portland, OR, USA), and NADPH was from Oriental Yeast Co. Ltd. (Tokyo, Japan). Trolox and 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPOL) were purchased from Aldrich (Milwaukee, WI, USA). All other reagents were of commercially highest purity. Pure water was freshly prepared with a Millipore Milli-Q Labo (Bedford, MA, USA).

Generation of $^1O_2$ by Photosensitization

A sample solution containing 14 μM UP, RB or MB and the spin trap with or without reductants in 20 mM sodium phosphate buffer, pH 7.4 (PB) was transferred to a quartz flat cell (Labotec) and irradiated (0.7 W/m^2) with visible light (tungsten bulb, Philips AP-12, 750 W) at room temperature for 2 min.

Measurement of Oxygen Radicals by ESR/Spin Trapping

X-Band ESR spectra of radical adducts were recorded with a JEOL JES-RE 1X spectrometer at 0.079 mT with 100 kHz field modulation. ESR signal heights of DMPO radical adducts were normalized with that of manganese oxide used as an external standard. The concentration of DMPO radical adducts was determined by comparing the double integrated value of the ESR spectrum of the DMPO adducts with that of 5 μM TEMPOL in PB.

Fenton Reaction

A 2 mM $FeSO_4 \cdot 7H_2O$ solution was prepared with an aqueous solution of 1.1 mM diethylenetriamine-N, N, N′, N″, N″-pentaacetic acid (DTPA), and diluted appropriately with water. Other reagents were prepared with PB. Sixty microliters of 1 mM H_2O_2 was added to 120 μl of a mixture containing various concentrations of $FeSO_4$ and 70 mM DMPO or DEPMPO, and stirred quickly. ESR spectra were recorded 1 min after the mixing.

HPLC-ECD Analysis of SA Trapping

2,3-DHBA and 2,5-DHBA generated from the photosensitization of UP in the presence of 32 mM SA were analyzed by HPLC with an electrochemical detector (ECD) (Coulochem II, ESA, USA) equipped with a Model 5011 analytical cell. HPLC was carried

out using a CCP & 8020 system (Tosoh, Tokyo, Japan) with a TSK-GEL Octyl 80-Ts (4.6 × 250 mm, Tosoh) reverse phase column. The potential of the first electrode was set at 30 mV, and that of the second at 300 mV in the oxidative mode. The HPLC mobile phase consisted of 30 mM citrate, 30 mM acetate and a 20% (v/v) aqueous methanol solution adjusted to pH 3.2 with NaOH. Elution was performed at a flow rate of 1.0 ml/min and at a column temperature of 43°C. 2,3-DHBA, 2,5-DHBA and catechol were separated completely under these conditions. Retention times of DHBAs were checked every time with authentic standards.

RESULTS

Remarkable Increase in the Signal Intensity of DMPO-OH in the Presence of Reductants

We investigated the effect of GSH, Trolox and NADPH on the formation of radical adducts of DMPO during the photosensitization of UP. A four-line ESR signal with hyperfine splitting constants corresponding to those of DMPO-OH ($a^N = 1.49$ mT, $a^H = 1.49$ mT)[13,20] increased remarkably in intensity in the presence of GSH, Trolox or ascorbic acid (Fig. 1) as observed with NADPH previously.[18] Concentrations of DMPO-OH formed in the presence of reductants were 2.7 μM for GSH, 2.2 μM for Trolox and 1.9 μM for NADPH while its concentration was 0.3 μM in the absence of reductant. As shown in Fig. 2, the presence of $^1O_2$ quencher, sodium azide, in the reaction system almost completely suppressed the appearance of signal of DMPO-OH. The replacement of 97% of H_2O with D_2O, which increases the lifetime of $^1O_2$, resulted in the increase in the initial rate of DMPO-OH generation by a factor of 1.8–2.5 (data not shown). The addition of ·OH scavengers, ethanol and sodium formate, resulted in a reduction in the signal intensity of DMPO-OH, and the appearance of signals of the α-hydroxyethyl radical (·$CH(CH_3)OH$) adduct ($a^N = 1.58$ mT, $a^H = 2.28$ mT)[20,21] and carbon dioxide anion radical (·CO_2^-) adduct ($a^N = 1.56$ mT, $a^H = 1.87$ mT)[20,21] of DMPO, respectively. These results were obtained with GSH, with ascorbic acid and with Trolox, and similar to the results observed with NADPH previously.[18]

A similar increase of DMPO-OH was observed with dihydrolipoic acid and 5-hydroxy-L-tryptophan (data not shown), and the enhancing effect of the reductants was also observed when RB or MB was used instead of UP as a photosensitizer, excluding the case of MB photosensitization in the presence of NADPH (Fig. 3). These results indicate that biological reductants including GSH and Trolox increased $^1O_2$-dependent formation of free ·OH in ESR/spin trapping with DMPO as well as NADPH,[18] and that this reaction does not depend on the structure of photosensitizers.

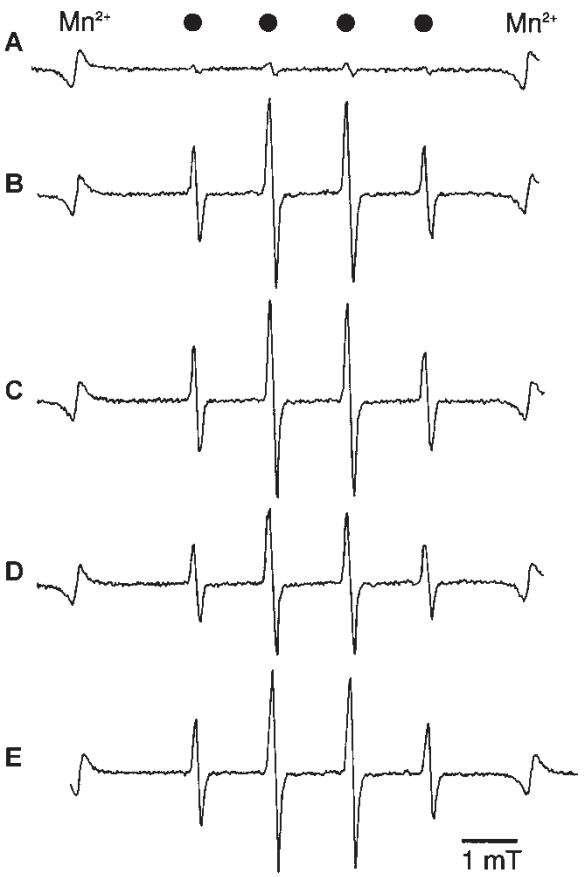

FIGURE 1 ESR spectra of DMPO radical adducts formed during UP photosensitization. Samples containing 14 μM UP and 47 mM DMPO in 20 mM PB, pH 7.4, were irradiated with visible light in the absence (A) or presence of GSH (B), Trolox (C), NADPH (D) or ascorbic acid (E). The concentration of GSH, Trolox and NADPH was 0.17 mM and that of ascorbic acid was 0.017 mM. Higher concentration of ascorbic acid decreased the signal because of reduction of the DMPO radical adduct to ESR silent form by excess ascorbic acid. Symbol ● indicates signal of DMPO-OH. ESR spectra were recorded at gain 200.

Formation of the ·OH Adduct of DEPMPO

To elucidate whether DMPO is involved in the $^1O_2$-dependent formation of free ·OH during UP photosensitization or not, spin trapping was performed using DEPMPO instead of DMPO. As shown in Fig. 4, an eight-line ESR signal was observed in the absence and presence of reductants. The hyperfine splitting constants ($a^P = 4.74$ mT, $a^N = 1.40$ mT, $a^{H(\beta)} = 1.37$ mT, $a^{H(\gamma)} = 0.04$ mT (6H)) and ratio of signal intensity corresponded to those of the ·OH adduct of DEPMPO (DEPMPO-OH).[22,23] The signal intensity of DEPMPO-OH was slightly greater in

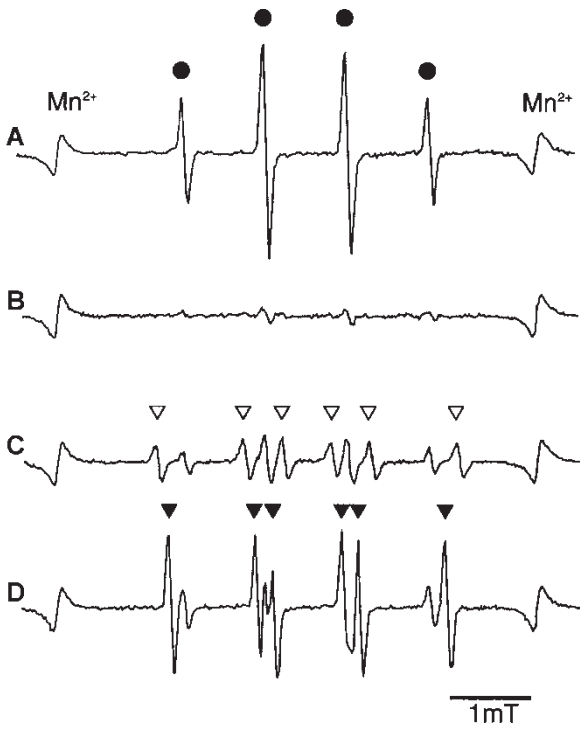

FIGURE 2 Effects of antioxidants on DMPO-OH formation during UP photosensitization in the presence of GSH. Samples containing 14 μM UP, 47 mM DMPO and 0.17 mM GSH in 20 mM PB, pH 7.4, were irradiated with visible light in the absence (A) or presence of 5 mM sodium azide (B), 2.8% ethanol (C) or 143 mM sodium formate (D). Symbols ●, ▽ and ▼ indicate signal of DMPO-OH, DMPO-$CH(OH)CH_3$ and DMPO-CO_2^-, respectively.

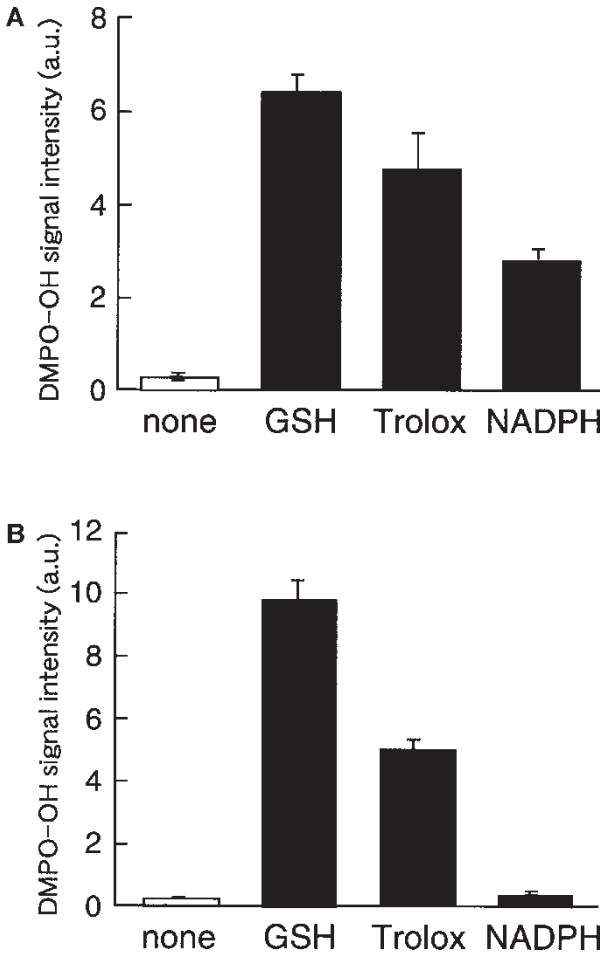

FIGURE 3 Effects of reductants on signal intensity of DMPO-OH formed during photosensitization of RB (A) and MB (B). Samples containing 14 μM RB or MB and 47 mM DMPO in 20 mM PB, pH 7.4, were irradiated with visible light in the absence or presence of 0.17 mM reductants as indicated. The values are the average of three experiments, and the bars indicate standard deviation.

the presence of reductants. Although the signal for the $O_2^{\cdot-}$ adduct of DEPMPO (DEPMPO-OOH) was superimposed on the signal of DEPMPO-OH both in the absence and in the presence of NADPH, the signal intensity of DEPMPO-OOH was greater in the presence of NADPH consistent with the report that NADPH reduces $^1O_2$ to $O_2^{\cdot-}$.[24] An unknown signal was superimposed on the DEPMPO-OH signal in the presence of Trolox, while only the DEPMPO-OH signal was observed in the presence of GSH.

To compare the increase in the signal intensity of DEPMPO-OH in the presence of reductants with that of DMPO-OH, the relationship between signal intensities of DEPMPO-OH and DMPO-OH formed with UP photosensitization was compared with that between the signal intensities of DEPMPO-OH and DMPO-OH formed by the Fenton reaction. As shown in Fig. 5, the effect of reductants on the formation of DEPMPO-OH was very small compared with that on the formation of DMPO-OH under conditions of UP photosensitization, considering the relationship obtained with ·OH generated by the Fenton reaction. This indicates that the increase in the signal intensity of the ·OH adduct caused by the reductants is very small when DEPMPO is used as a spin trap during the photosensitization of UP.

DMPO-mediated Increase in the Signal Intensity of DEPMPO-OH

The small increases in the signal intensity of DEPMPO-OH compared with DMPO-OH suggest the participation of DMPO in the $^1O_2$-dependent generation of ·OH during the photosensitization of UP in the presence of reductants. To confirm this possibility, we examined the effect of DMPO on the formation of DEPMPO-OH during spin trapping with DEPMPO. When DMPO was added to the DEPMPO-spin trapping system under UP photosensitization in the presence of GSH, the ESR signal of DEPMPO-OH was enhanced significantly

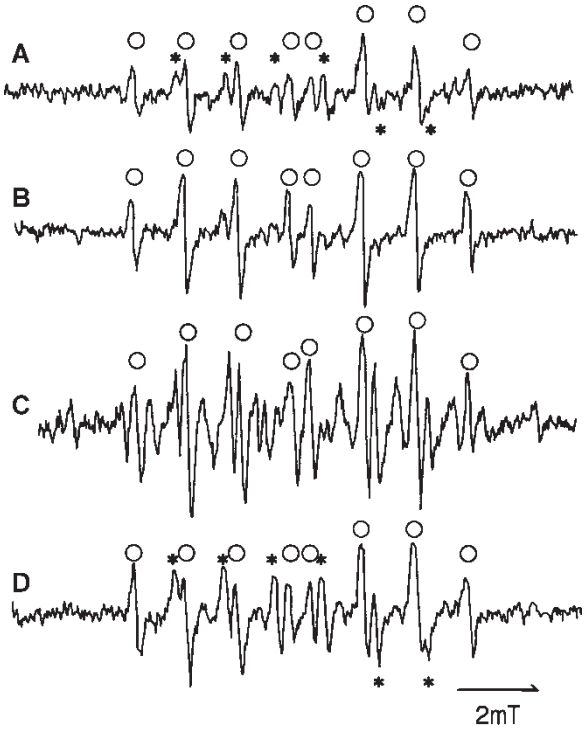

FIGURE 4 ESR spectra of DEPMPO adducts formed during UP photosensitization. Samples containing 14 μM UP and 47 mM DEPMPO in 20 mM PB, pH 7.4, were irradiated with visible light in the absence (A) or presence of 0.17 mM GSH (B), Trolox (C) or NADPH (D). ESR spectra were recorded at gain 2500. Symbol ○ indicates signal of DEPMPO-OH. Peaks indicated with * arose from spectral lines of DEPMPO-OOH which did not overlap with spectral lines of DEPMPO-OH.

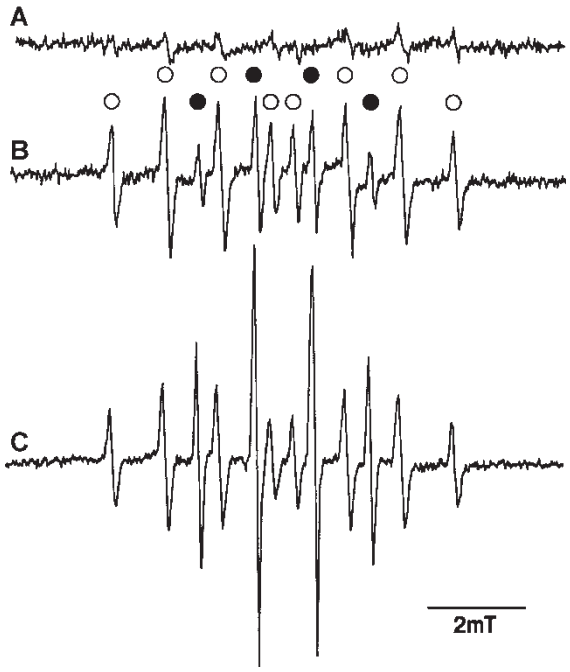

FIGURE 6 Effect of addition of DMPO to the spin trapping system with DEPMPO on the formation of ·OH adducts of DEPMPO and DMPO. Samples containing 14 μM UP, 47 mM DEPMPO and 0.17 mM GSH in 20 mM PB, pH 7.4, were irradiated with visible light (A). The samples with the same composition as (A) were also irradiated in the presence of 20 mM (B) and 47 mM DMPO (C). ESR spectra for (A) and (B) were recorded at gain 630, and that of (C) at gain 400. Symbols ○ and ● indicate DEPMPO-OH and DMPO-OH signals, respectively.

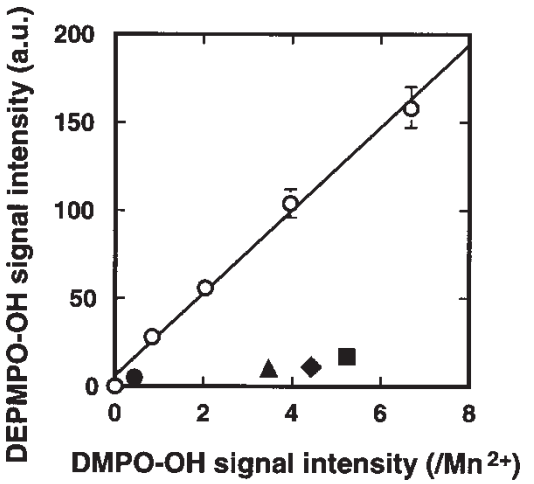

FIGURE 5 Relationship between the signal intensities of DMPO-OH and DEPMPO-OH. Samples containing 0.3 mM H_2O_2 and 47 mM DMPO or DEPMPO were incubated with 0, 5.6, 11, 22 or 33 μM $FeSO_4$ for 2 min at room temperature (○). Samples containing 14 μM UP and 47 mM DMPO or DEPMPO in 20 mM PB, pH 7.4, were irradiated with visible light in the absence (●), or presence of 0.17 mM GSH (◆), NADPH (▲) or Trolox (■). The values are the average of three experiments, and the bars indicate standard deviation. The size of the bars for UP photosensitization was within the size of the symbols. ESR spectrum of DEPMPO-OH was recorded without a Mn^{2+} external standard, because signal of Mn^{2+} overlaps with that of DEPMPO-OH. Signal intensity for DEPMPO-OH was expressed as relative value of incorrected signal height.

depending on the concentration of DMPO, in addition to the increase in the signal intensity of DMPO-OH (Fig. 6). Similar results were obtained when NADPH and Trolox were used instead of GSH (data not shown). These results strongly indicate that the $^1O_2$-dependent formation of ·OH in the presence of reductants is mediated by DMPO.

Increase of ·OH-dependent Hydroxylation of SA Caused by DMPO

Radical adducts formed by trapping with nitrone lose their paramagnetism via oxidation or reduction. Therefore, the formation of ·OH was assessed with another index, hydroxylation of SA. The attack of ·OH on SA generates 2,3-DHBA and 2,5-DHBA as major products, and catechol as a minor product,[25–32] while the attack of $^1O_2$ on SA generates 2,5-DHBA as a major product with negligible 2,3-DHBA formation.[15,33] Thus, the production of 2,3-DHBA appears to be a useful marker of the generation of ·OH. Figure 7 shows the effect of DMPO on 2,3-DHBA production during the photosensitization of UP in the presence of GSH. Both ethanol and sodium azide inhibited this

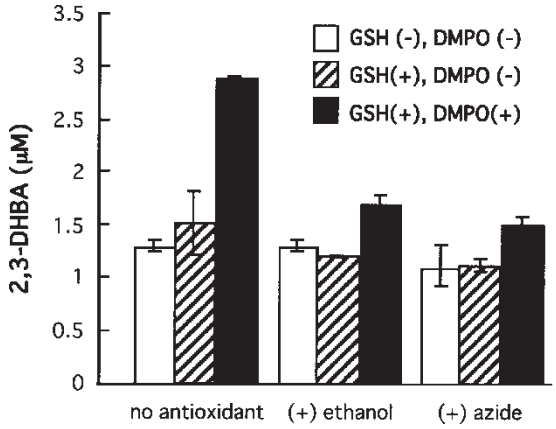

FIGURE 7 Effects of DMPO and antioxidants on the formation of 2,3-DHBA. A measure of 14 μM UP and 32 mM SA in 20 mM PB, pH 7.4, containing indicated compounds was irradiated with visible light in the absence and presence of 2.8% ethanol or 5 mM sodium azide. The concentrations of GSH and DMPO were 0.17 mM and 47 mM, respectively. The values are the average of three experiments, and the bars indicate standard deviation.

enhancement (Fig. 7), indicating that the increase in 2,3-DHBA caused by DMPO depends on free ·OH and $^1O_2$.

Neither ethanol nor sodium azide inhibited the production of 2,3-DHBA in the absence of DMPO (Fig. 7), suggesting that the contributions of ·OH and $^1O_2$ are very small in the absence of DMPO. The exclusion of GSH from the reaction system in the absence of DMPO decreased the amount of 2,3-DHBA by less than 15%. No more decrease was observed by addition of either ethanol or NaN_3, indicating that the production of remaining part of 2,3-DHBA results from $^1O_2$- and ·OH-independent mechanism, such as oxidation of SA by photosensitizer in the excited state. The ·OH-dependent formation of 2,3-DHBA was quantified by subtracting the amount of 2,3-DHBA formed without DMPO from that formed in the presence of DMPO. As shown in Table I, the addition of DMPO increased 2,3-DHBA production to a similar extent for all reductants (about 1.5 μM). The addition of either ethanol or sodium azide reduced production. These results support the conclusion that GSH, Trolox and NADPH increase $^1O_2$-dependent formation of free ·OH through a DMPO-mediated mechanism.

DISCUSSION

GSH, ascorbic acid, Trolox, dihydrolipoic acid and 5-hydroxy-L-tryptophan significantly increased the signal intensity of DMPO-OH during the photosensitization of UP. This ESR signal observed in the presence of reductants decreased on addition of a $^1O_2$ quencher or ·OH scavenger, suggesting that the generation of DMPO-OH is related to the $^1O_2$-dependent formation of free ·OH. This production of ·OH was $O_2^{\cdot-}$-independent, because neither superoxide dismutase nor catalase affected the signal strength of DMPO-OH (data not shown). Similar results have been reported with NADPH,[18] suggesting that the increase in the signal intensity of DMPO-OH due to $^1O_2$-dependent formation of ·OH is probably a common occurrence in the presence of biological reductants. Furthermore, the increase was observed during photosensitization using three different types of photosensitizers, suggesting that photosensitizers themselves are not associated with the $^1O_2$-dependent formation of ·OH.

It was clarified that the level of production of the ·OH adduct of DEPMPO was much lower than that of the ·OH adduct of DMPO in the photosensitization of UP in the presence of reductants. The very low level of DEPMPO-OH despite the high reaction rate constant of DEPMPO with ·OH (7.1×10^9/M/s)[22] compared to that of DMPO with ·OH (3.4×10^9/M/s),[22,32] suggests that the formation of ·OH is dependent on DMPO. This is supported by the observation that the ESR signal of DEPMPO-OH strengthened depending on the concentration of DMPO added to the spin trapping system with DEPMPO. Similarly, the addition of DMPO increased production of 2,3-DHBA, a product of the reaction of SA with ·OH, during the photosensitization of UP in the presence of reductants. This increase was inhibited by the addition of a ·OH scavenger or $^1O_2$ quencher, indicating the participation of $^1O_2$-mediated formation of ·OH.

Spin trapping using 47 mM DMPO resulted in the formation of 2–3 μM of DMPO-OH during UP photosensitization in the presence of reductants, whereas the same concentration of DMPO increased 2,3-DHBA production by about 1.5 μM under the corresponding conditions in the presence of 32 mM SA. The production of the detectable amount

TABLE I Inhibition of the DMPO-dependent 2,3-DHBA formation by antioxidants*

Antioxidants	Concentration	GSH	Trolox	NADPH
None		1.4 (100)†	1.6 (100)	1.5 (100)
Ethanol	2.8%	0.5 (35.7)	0.4 (25.0)	0.5 (33.3)
Sodium azide	5 mM	0.4 (28.6)	0.1 (6.3)	0.6 (40.0)

*Samples containing 14 μM UP, 32 mM SA and 0.17 mM reductants were irradiated with visible light in the absence or presence of 47 mM DMPO. DMPO-dependent 2,3-DHBA (μM) was obtained by subtracting the amount of 2,3-DHBA produced in the absence of DMPO from that produced in its presence.
†The values in parentheses are percentages with the value for no antioxidants 100%.

of 2,3-DHBA despite of the presence of DMPO in the reaction system may be due to ten times higher rate constant of the reaction of ·OH with SA (1.2×10^{10}/M/s)[34] than the rate constant of the reaction with DMPO. Although it is difficult to compare directly the amounts of products obtained by different analytical techniques for ·OH measurements, the amount of 2,3-DHBA formed seems reasonable compared to that of DMPO-OH considering the concentrations of traps (DMPO and SA) and the rate constants of reactions of those with ·OH.

Photosensitization is applied to photodynamic therapy for malignant carcinoma. A number of new effective photosensitizers have been developed.[35–37] The ESR/spin trapping technique is generally utilized to assess their ability to generate oxygen radical. However, the artificial formation of ·OH resulting from the reaction of DMPO with $^1O_2$ has been proposed in the photosensitizing reaction of micellar RB,[16] bacteriochlorin a[21] and C-phycocyanin,[38] after it was first suggested by Feix and Kalyanaraman[15] for merocyanin 540. Both $^1O_2$ and ·OH modify biological molecules and damage cells.[1–12] Some $^1O_2$ is quenched to molecular oxygen in the ground state, for its short lifetime (approximately 2 μs).[39] In this case, $^1O_2$ does not contribute to the modification of any biological molecules. This means that the accurate determination of active species is important for the assessment of photosensitizers.

The present study demonstrated that the signal intensity of DMPO-OH increased due to the DMPO-mediated formation of ·OH from $^1O_2$ in the presence of biological reductants. GSH, NADPH, ascorbic acid and tocopherol homologues are present *in vivo* at various levels; e.g. the *in vivo* concentration of GSH is known to range from several μM for blood plasma to several mM for various tissue cells.[32] A remarkable enhancement of DMPO-OH was observed in the presence of reductants at 0.17 mM in this study; this means that similar artificial reactions very likely occur in biological samples. This study also indicates that DMPO is not suitable for measuring the generation of ·OH during photosensitization in such biological samples, and that spin trapping techniques using DEPMPO or SA are appropriate for this purpose. This should be an important notice in spin trapping with DMPO, together with ·OH-independent formation of DMPO-OH by nucleophilic addition of H_2O to DMPO in the presence of iron ions, which was previously reported by Makino *et al*.[40]

The $^1O_2$- and spin trap-mediated ·OH generation was prevented by the used of DEPMPO instead of DMPO. As DEPMPO is an analogue of DMPO whose one methyl group at C5 position is replaced by a diethoxyphosphoryl group, a strong electron-withdrawing group, electrophilic reaction of $^1O_2$ with nitrone spin trap may be involved in the ·OH generation. However, the role of reductants in the increased generation of ·OH is, at present, unclear. Bilski *et al*.[16] speculated that the addition of $^1O_2$ to DMPO yields hydroperoxide, which produces ·OH by reductive cleavage of O—O bond. The enhancement of this cleavage by reductants may be one possibility. The mechanism of ·OH enhancement is to be clarified with further experiments.

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