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Original Research Communication

Singlet Oxygen-Dependent Hydroxyl Radical Formation during Uroporphyrin-Mediated Photosensitization in the Presence of NADPH

KEIZO TAKESHITA,[1] CLAUDIO A. OLEA-AZAR,[1,3] MICHIKO MIZUNO,[1,2] and TOSHIHIKO OZAWA[1]

ABSTRACT

The conversion of singlet oxygen ($^1O_2$) to hydroxyl radical ($^{\cdot}OH$) during photosensitization of uroporphyrin (UP) in the presence of NADPH was examined by a spin-trapping technique with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). Significant electron spin resonance (ESR) signals of DMPO-OH adduct were observed during irradiation of the UP-NADPH system with visible light. Scavengers of $^{\cdot}OH$ reduced the signal intensity to 3–30% of control, indicating that more than 70% of DMPO-OH results from freely diffusing $^{\cdot}OH$. The ESR signal was almost completely lost when quenchers of $^1O_2$ were added, and was enhanced when the amount of deutrated solvent was increased. The appearance of $^1O_2$, as determined by the oxidation of 2,2,6,6-tetramethyl-4-piperidone (TEMPD), was delayed with an increase in the concentration of NADPH, whereas the production of $^{\cdot}OH$ was upregulated. These observations indicate that conversion of $^1O_2$ to $^{\cdot}OH$ occurs quickly in the presence of NADPH. Hydrogen peroxide (H_2O_2) was produced $^1O_2$-dependently during irradiation of UP in the presence of NADPH. However, neither catalase nor desferrioxamine decreased the DMPO-OH signal, and addition of H_2O_2 did not increase the signal. SOD increased the signal only slightly. These results suggest that the production of $^{\cdot}OH$ from $^1O_2$ involves neither superoxide anion radical nor H_2O_2. Antiox. Redox Signal. 2, 355–362.

INTRODUCTION

PHOTOSENSITIZERS SUCH AS PORPHYRIN DERIVATIVES are used as agents for photodynamic therapy (Calzavara-Pinton *et al.*, 1996) but are causative of porphyrias (Brun and Sanberg, 1991). The therapeutic effect and the tissue damage are explained by the generation of reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), during photodynamic reactions and their successive reactions with biologically important molecules. The pathways for generating reactive oxygen species usually fall into two major categories: (1) type I reactions involving electron or hydrogen transfer between the substrate and the triplet excited sensitizer, with generation of free radicals and other reactive intermediates, *e.g.*, $O_2^{\cdot -}$, $^{\cdot}OH$, and H_2O_2, and (2) type II reactions involving energy transfer from the triplet sensitizer to oxygen, ac-

[1]Bioregulation Research Group, National Institute of Radiological Sciences, Chiba 263-8555, Japan.
[2]Kyoritsu College of Pharmacy, Tokyo 105-8512, Japan.
[3]Present address: Faculty of Science and Pharmacy, University of Chile, Olivos 1007, Independencia, Casila 233 Santiago 1, Chile.

companying the formation of $^1O_2$. Formation of $O_2^{\cdot -}$ was demonstrated during the reaction of $^1O_2$ with NAD(P)H by monitoring the reductions of benzoquinone (Peters and Rodgers,1981) and *p*-nitrotetrazolium blue (Inoue *et al.*, 1984). $^1O_2$-mediated formation of H_2O_2 was reported in the presence of SOD (Menon *et al.*, 1989) and NADPH (Bodaness and Chan, 1977). Furthermore, Buettner (1985) demonstrated $^1O_2$-mediated formation of ·OH in the presence of cysteine. These reports suggest that conversion of $^1O_2$ to other ROS is possible in the presence of certain biological substances. Reaction manners and biological effects vary depending on the kind of ROS (Ryter and Tyrrell, 1998; Halliwell and Gutteridge, 1999). Nevertheless, how $^1O_2$ is converted to other ROS is not fully understood. In the present study, we studied the conversion of $^1O_2$ to highly toxic ·OH in the presence of NADPH in a uroporphyrin (UP) photosensitizing system and demonstrated that ·OH is generated without the intermediates $O_2^{\cdot -}$ or H_2O_2.

MATERIALS AND METHODS

Materials

Uroporphyrin I dihydrochloride was obtained from Porphyrin Products (Logan, UT). 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Labotec (Tokyo). NADPH was obtained from Kohjin Co., Ltd. (Tokyo). Catalase (Bovine Liver), superoxide dismutase (SOD, Bovine Erythrocyte), horseradish peroxidase (type VI), 2,2,6,6-tetramethyl-4-piperidone hydrochloride (TEMPD) and desferrioxamine mesylate (DFO) were obtained from Sigma (St. Louis, MO). Deuterium oxide (D_2O) was purchased from Aldrich (Milwaukee, WI). Pure water was freshly prepared with a Millipore Milli-Q Labo (Bedford, MA). All other reagents were of the highest purity commercially available.

Electron spin resonance measurements

Oxygen radicals were determined by spin-trapping method with DMPO as the trapping agent, and TEMPD was used for the determination of $^1O_2$. The detection of $^1O_2$ is based on the oxidation of TEMPD to corresponding nitroxide radical, 2,2,6,6-tetramethyl-4-piperidone-*N*-oxyl (TEMPON) (Lion *et al.*, 1976; Moan and Wold, 1979). A sample solution was transferred to a quartz flat cell (Labotec, Tokyo) and irradiated with visible light at room temperature (24–25°C). X-band ESR spectra were recorded with a JEOL JES RE-1X spectrometer at 0.079 mT of 100 kHz field modulation. Manganese oxide was used as an internal standard.

Measurement of H_2O_2

Photodynamic generation of H_2O_2 was assayed according to the method of Frew *et al.* (1983) with a slight modification. In this method, H_2O_2 oxidatively couples with 4-aminoantipyrine and phenol to yield a quinoneimine chromogen with a maximum absorption at 505 nm. A 2.4-ml volume of sample solution in a quartz cubet was irradiated with visible light for 10 min at room temperature (24–25°C). To the solution was added 0.4 ml of a reagent which contains 0.5 wt/vol % 4-aminoantipyrine, 1.2 wt/vol % phenol, 0.1 μM horseradish peroxidase, and 12.5 μM H_2O_2. The sample solution incubated in the dark was used as a negative control. The absorbance of the resulting solution was measured at 505 nm. The concentration of H_2O_2 was determined with spectrophotometrically standardized H_2O_2 (ϵ = 43.5 $M^{-1}cm^{-1}$ at 240 nm).

Light source

A 750 W tungsten bulb (Philips AP-12) equipped with a reflector and a condensing lens was used as a visible light source. Using a model IL1400A radiometer/photometer with a SL021/FQI detector (International Light, Inc., Newburyport, MA), the light intensity was determined to be 0.7 W/m^2 for spin-trapping experiments and 10 W/m^2 for H_2O_2 determination experiments.

RESULTS

Irradiation of UP with visible light in the presence of NADPH and DMPO produced a four-line (1:2:2:1) ESR spectrum with hyperfine

splittings ($a^N = a^H = 1.49$ mT) characteristic of the ·OH adduct of DMPO (DMPO-OH) (Fig. 1A) (Buettner, 1987; Ozawa and Hanaki, 1991). The signal intensity of DMPO-OH was obviously reduced when NADPH was excluded from the reaction mixture (Fig. 1B), and no signal was observed on incubation in the dark (data not shown). The addition of ethanol and sodium formate during irradiation resulted in a reduction in the intensity of signal of DMPO-OH and the appearance of a six-line ESR signal which was assigned to the $\cdot CH(OH)CH_3$ adduct of DMPO ($a^N = 1.59$ mT, $a^H = 2.29$ mT) and the $\cdot CO_2^-$ adduct of DMPO ($a^N = 1.57$ mT, $a^H = 1.87$ mT), respectively (Fig. 1C, D) (Buettner, 1987). Addition of ethanol, sodium formate, or dimethyl sulfoxide (DMSO) reduced the signal intensity to 3–30% of control (Table 1). These observations suggest that more than 70% of DMPO-OH results from freely diffusing ·OH produced during irradiation of UP and NADPH, but not from decay of the $O_2\cdot^-$ adduct of DMPO. Similar results were obtained when NADH was used instead of NADPH.

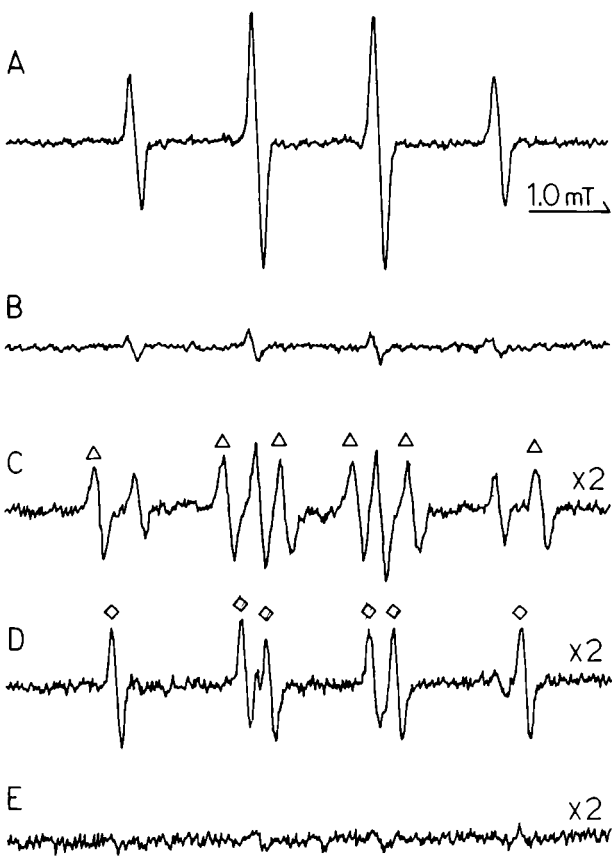

Fig. 1. ESR spectra of DMPO spin adducts formed during irradiation of UP. (**A**) 14 μM UP, 167 μM NADPH, and 48 mM DMPO in 20 mM phosphate buffer, pH 7.4, was irradiated with visible light for 2 min. (**B**) Same conditions except without NADPH. Samples containing 600 mM ethanol (**C**), 143 mM sodium formate (**D**), and 5 mM NaN_3 (**E**) were irradiated in the presence of NADPH. (△) $\cdot CH(OH)CH_3$ adduct of DMPO; (◇) $\cdot CO_2^-$ adduct of DMPO.

The signal of DMPO-OH was almost completely lost on addition of quenchers of $^1O_2$ such as sodium azide, L-histidine, and 1,4-diazabicyclo[2.2.2]octane (DABCO) (Table 1). No $N_3\cdot$ adduct of DMPO was observed (Fig. 1E). To obtain evidence of the involvement of $^1O_2$ in the production of ·OH, the formation of DMPO-OH was examined in the deuterated solvent where the half-life of $^1O_2$ is known to be elongated. Replacement of 97% of H_2O with D_2O clearly increased both the rate and extent of DMPO-OH formation (Fig. 2). These observations strongly indicate that $^1O_2$ is an intermediate of ·OH.

Role of $^1O_2$ in the ·OH formation was further clarified by examining the oxidation of TEMPD by $^1O_2$. Signal heights for TEMPON formation in the presence of different concentrations of NADPH were measured along with time and were superimposed on those for DMPO-OH formation obtained under the same concentrations of UP and NADPH (Fig. 3). In the absence of NADPH, the signal of TEMPON increased with increase of irradiation time. This signal significantly decreased when irradiation was performed in the presence of quenchers of $^1O_2$ such as NaN_3 and L-histidine, although ·OH scavengers, catalase, and SOD had less or no effect (Table 1), indicating that oxidation of TEMPD to TEMPON is specific to $^1O_2$ under this condition. The presence of NADPH delayed the appearance of TEMPON signal (Fig. 3). The lag time increased with the concentration of NADPH, whereas the rate of formation of DMPO-OH increased with the concentration of NADPH. These observations imply that $^1O_2$ is converted to ·OH NADPH dependently, and that the apparent reaction rate constant of $^1O_2$ for NADPH is much larger than the rate constant of $^1O_2$ for TEMPD ($4–8 \times 10^7\ M^{-1}\cdot s^{-1}$) (Lion *et al.*, 1976; Moan and Wold, 1979).

One-electron reduction of $^1O_2$ by NAD(P)H (Peters and Rodgers, 1981; Inoue *et al.*, 1984) has been proposed in chemical reactions. This reaction gives $O_2\cdot^-$ as a product. On the other

TABLE 1. EFFECTS OF INHIBITORS ON DMPO-OH FORMATION AND H_2O_2 PRODUCTION IN A UP-NADPH PHOTOSENSITIZING SYSTEM AND ON TEMPON PRODUCTION IN A UP-PHOTOSENSITIZING SYSTEM

Inhibitors	*Concentrations*	*DMPO-OH (relative signal intensity) in UP-NADPH-light system*[a]	*TEMPON (relative signal intensity) in UP-light system*[b]	*H_2O_2 (μM) in UP-NADPH-light system*[c]
None		1.89 ± 0.26 (100)	0.396 ± 0.084 (100)	51.4 ± 7.6 (100)
Ethanol	600 m*M*	0.53 ± 0.03 (27)	0.393 ± 0.020 (99)	ND
DMSO	143 m*M*	0.33 ± 0.14 (18)	0.276 ± 0.031 (70)	ND
Sodium formate	143 m*M*	0.06 ± 0.03 (3)	0.275 ± 0.022 (67)	ND
NaN_3	5 m*M*	0.06 ± 0.02 (3)	0.135 ± 0.025 (34)	3.2 ± 0.5[d] (6)
L-Histidine	5 m*M*	0.06 ± 0.02 (3)	0.000 ± 0.000 (0)	ND
DABCO	5 m*M*	0.26 ± 0.01 (14)	0.295 ± 0.025 (74)	ND
DFO	20 μM	2.03 ± 0.21 (107)	0.282 ± 0.087 (71)	59.0 ± 1.6[e] (115)
SOD	100 U/ml	2.93 ± 0.59 (155)	0.425 ± 0.060 (107)	ND
Catalase	280 U/ml	2.43 ± 0.40 (129)	0.391 ± 0.027 (99)	0.0 ± 0.1[f] (0)
SOD + catalase	100 U/ml + 280 U/ml	2.70 ± 0.20 (143)	ND	ND

Each value is the mean ± SD for triplicate experiments. Numbers in parentheses are the percentage to the value for no inhibitor. ND, Not determined.
[a]Sample solutions containing 14 μM UP, 167 μM NADPH, 48 m*M* DMPO, and indicated concentrations of inhibitor were irradiated.
[b]Sample solutions containing 14 μM UP, 50 m*M* TEMPD, and indicated concentrations of inhibitor were irradiated.
[c]Sample solutions containing 2.5 μM UP, 0.17 m*M* NADPH, and indicated concentrations of inhibitor were irradiated.
[d]6 m*M* NaN_3 was used.
[e]17 μM desferrioxamine was used.
[f]120 U/mL catalase was used.

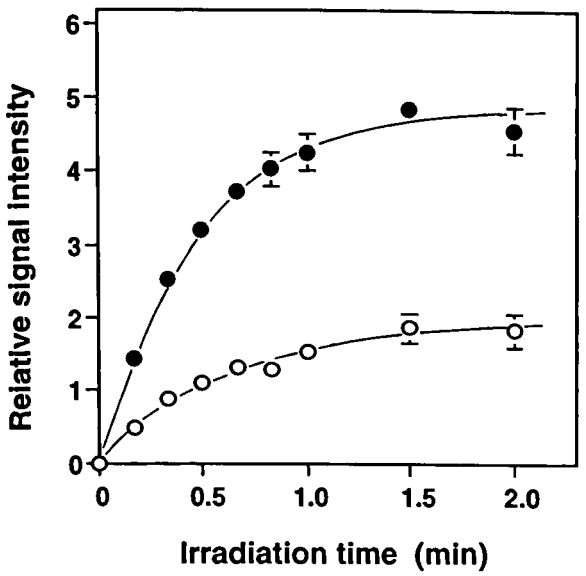

Fig. 2. Enhancement of DMPO-OH formation in deuterated solvent. Irradiated with visible light was 14 μM UP, 167 μM NADPH, and 48 mM DMPO in 5 mM phosphate buffer, pH 7.4, prepared with H_2O (○) or 97% D_2O (●). The ratio of the signal intensity of the second low-field peak to that of the manganese peak (internal standard) was plotted. Data are presented as mean values of duplicate experiments. Error bars indicate deviations.

hand, two-electron reduction of $^1O_2$ to H_2O_2 by NADPH was also suggested in a hematoporphyrin photodynamic system (Bodaness and Chan, 1977). $O_2^{\cdot -}$ and/or H_2O_2 may produce ·OH through metal-catalyzed reactions. To confirm whether or not the generation of ·OH observed in the present study occurs through $O_2^{\cdot -}/H_2O_2$, effects of DFO, a Fe-chelator, SOD, and catalase on DMPO-OH formation were examined in a UP-NADPH photodynamic system. DFO and catalase did not reduce the DMPO-OH formation at the concentration indicated in Table 1. SOD somewhat enhanced the DMPO-OH formation, but the increase was not inhibited by addition of catalase, indicating that the effect does not result from H_2O_2 formation enhanced by SOD. Catalase was not effective even at a higher concentration (21,000 U/ml). At a higher concentration (0.7 mM), DFO decreased the rate of DMPO-OH formation (data not shown).

To clarify the reaction pathway further, the amount of H_2O_2 produced during the UP-NADPH photodynamic reaction was determined. As shown in Table 1, about 50 μM of H_2O_2 was produced in the presence of 2.5 μM

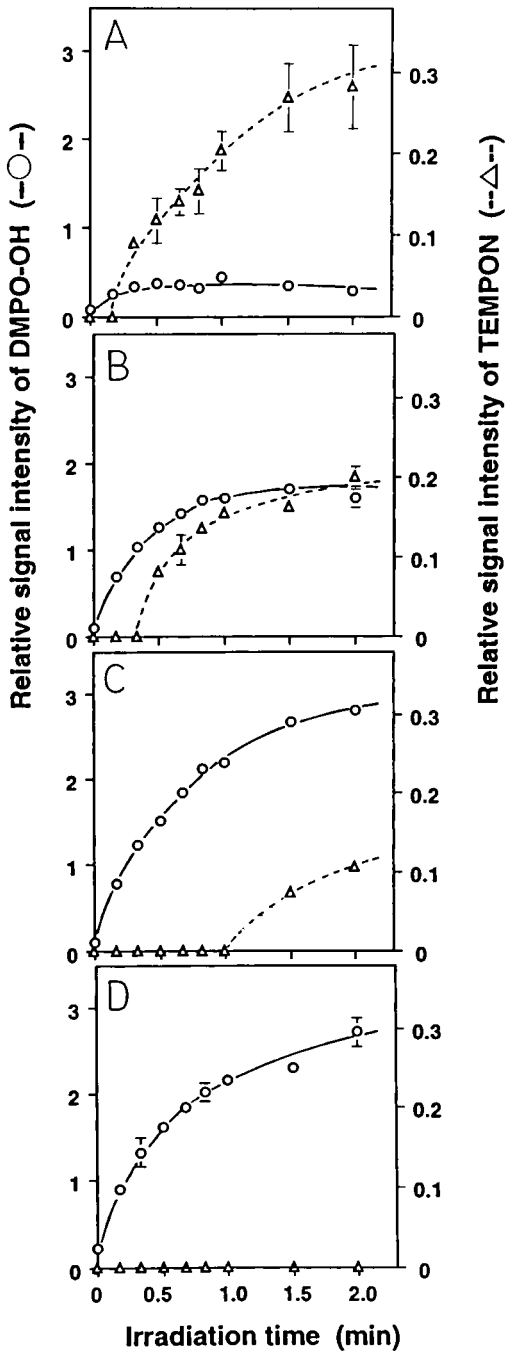

Fig. 3. Effects of NADPH on the formations of DMPO-OH and TEMPON during photosensitization of UP. Irradiated with visible light in the presence of either 48 mM DMPO or 50 mM TEMPD were 14 μM UP and various concentrations of NADPH in 20 mM phosphate buffer, pH 7.4. Concentrations of NADPH were (**A**) 0 μM, (**B**) 7 μM, (**C**) 33 μM, and (**D**) 167 μM. The second low-field peak for DMPO-OH and the first low-field peak for TEMPON were used to obtain relative signal intensities. Data are presented as mean values of duplicate experiments. Error bars indicate deviations.

UP and 2 m*M* NADPH. SOD exhibited no effect on the production of H_2O_2, while catalase or NaN_3 completely inhibited it. DFO increased the rate of formation of H_2O_2 little. To confirm the involvement of H_2O_2 in the production of ·OH, UP, and UP-NADPH systems were irradiated in the presence of 0.63 m*M* H_2O_2. As shown in Fig. 4, the formation of DMPO-OH was not upregulated, even in the presence of H_2O_2. This result strongly indicates that conversion of H_2O_2 to ·OH hardly occurs, even if H_2O_2 is produced during irradiation, and suggests that the enhancing effect of SOD and reducing effect of DFO on DMPO-OH formation is a side-effect of these reagents; at high concentration, SOD is known to induce Cu-catalyzed ·OH production (Yim *et al.* 1990), and DFO is known to scavenge ·OH (Halliwell, 1989).

DISCUSSION

The present study clearly demonstrated that $^1O_2$ is an intermediate in the production of ·OH during the photodynamic reaction of UP in the presence of NADPH. $^1O_2$-dependent production of ·OH has been reported in various photodynamic reaction systems. The following three pathways were proposed:

$$^1O_2 \xrightarrow{e^-} O_2^{\cdot -} \xrightarrow{e} H_2O_2 \xrightarrow{\text{Metals}} {\cdot OH} \xrightarrow{\text{DMPO}} \text{DMPO-OH} \quad (1)$$

$$^1O_2 \rightarrow [\text{DMPO-}^1O_2] \xrightarrow{H^+} \text{DMPO-OH} + {\cdot OH} \xrightarrow{\text{DMPO}} \text{DMPO-OH} \quad (2)$$

$$^1O_2 \xrightarrow{-SH} -SOOH \rightarrow -SO^{\cdot} + {\cdot OH} \xrightarrow{\text{DMPO}} \text{DMPO-OH} \quad (3)$$

$^1O_2$-mediated $O_2^{\cdot -}$ and H_2O_2 production have been reported in the presence of NADPH (Bodaness and Chan, 1977; Peters and Rodgers, 1981). If transition metal ion is present, ·OH can be formed from H_2O_2 (pathway 1). In the present study, addition of H_2O_2 to the reaction mixture did not increase the intensity of DMPO-OH signal either in the presence or absence of NADPH, although $^1O_2$-mediated production of H_2O_2 was observed during the UP-NADPH photodynamic reaction. Neither catalase nor DFO affected NADPH-enhanced DMPO-OH production. These observations show that ·OH is derived from a pathway other than pathway 1. Pathway 2 has been proposed in photosensitizing reaction of bacteriochlorin a (Hoebeke *et al.*, 1997), merocyanine 540 (Feix and Kalyanaraman, 1991), and C-phycocyanin (Zhang *et al.*, 1999). Formation of DMPO-OH through this pathway is involved in the decomposition of a product of the reaction of DMPO with $^1O_2$. However, this possibility can be ruled out in the present case for the following reasons: (1) more than 70% of the DMPO-OH signal was lost in the presence of ·OH scavengers, although one-half of DMPO-OH results from decay of the reaction product [DMPO-$^1O_2$], and the rest from free ·OH in pathway 2; and (2) if the reaction of $^1O_2$ with DMPO gives DMPO-OH signal, an equivalent signal of DMPO-OH should be observed regardless of the presence of NADPH, assuming that NADPH does not affect the generation of $^1O_2$. Buettner (1985) observed $^1O_2$-dependent formation of DMPO-OH in a photodynamic reaction of hematoporphyrin derivative in the presence of cysteine and proposed that ·OH is

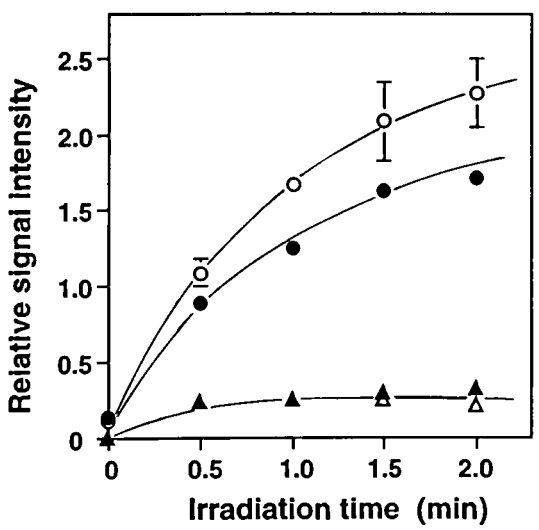

Fig. 4. Effect of H_2O_2 addition on the formation of DMPO-OH during photosensitization of UP. Samples of 14 μM UP, 167 μM NADPH, and 48 m*M* DMPO in 20 m*M* phosphate buffer, pH 7.4, were irradiated with visible light in the presence (●) or absence of 0.63 m*M* H_2O_2 (○). Samples of 14 μM UP and 48 m*M* DMPO in 20 m*M* phosphate buffer, pH 7.4, were irradiated with visible light in the presence (▲) or absence (△) of 0.63 m*M* H_2O_2. The ratio of signal intensity of the second low-field peak to that of the manganese peak (internal standard) was plotted. Data are presented as mean values of duplicate experiments. Error bars indicate deviations.

produced by homolytical cleavage of -SOOH, a product of the reaction of thiol with $^1O_2$. We could not determine whether or not a similar reaction occurs in the present system, but results obtained here indicate the existence of unknown pathways.

In conclusion, we obviously demonstrated $^1O_2$-derived ·OH formation in a UP-NADPH photodynamic reaction system and suggested the occurrence of other pathways for conversion of $^1O_2$ to ·OH than metal-catalyzed reactions of $O_2^{\cdot -}$ and H_2O_2 derived from $^1O_2$, although details of the pathway are still unknown. NADPH and NADH are continuously produced in the cells. This suggests that ·OH generated from $^1O_2$ contributes at least in part to the mechanism of porphyria phototoxicity and photodynamic therapeutic effect.

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ABBREVIATIONS

DABCO, Diazabicyclo[2.2.2]octane; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DFO, desferrioxamine mesylate; D_2O, deuterium oxide; DMSO, dimethyl sulfoxide; ESR, electron spin resonance; H_2O_2, hydrogen peroxide; $^1O_2$, singlet oxygen; ·OH, hydroxyl radical; $O_2^{\cdot -}$, superoxide anion radical; ROS, reactive oxygen species; SOD, superoxide dismutase; TEMPD, 2,2,6,6-tetramethyl-4-piperidone hydrochloride; TEMPON, 2,2,6,6-tetramethyl-4-piperidone-*N*-oxyl; UP, uroporphyrin.

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Address reprint requests to:
Dr. Toshihiko Ozawa
Bioregulation Research Group
National Institute of Radiological Sciences
Chiba 263–8555, Japan

E-mail: ozawa@nirs.go.jp

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