Cancer Letters, 25 (1985) 297—304
Elsevier Scientific Publishers Ireland Ltd.

HYDROGEN PEROXIDE AND HYDROXYL FREE RADICAL PRODUCTION BY HEMATOPORPHYRIN DERIVATIVE, ASCORBATE AND LIGHT

GARRY R. BUETTNER and MICHAEL J. NEED[a]

Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, NC 27709 and [a]Wabash College, Crawfordsville, IN 47933 (U.S.A.)

(Received 20 August 1984)
(Revised version received 2 November 1984)
(Accepted 13 November 1984)

SUMMARY

Photofrin II, a commercial preparation of a hematoporphyrin derivative containing a high proportion of the active di-hematoporphyrin ether, was found to convert oxygen to hydrogen peroxide and hydroxyl free radicals in the presence of ascorbate and light. In addition, a light-dependent increase in the ascorbate free radical concentration was observed in hematoporphyrin derivative/ascorbate solutions with subsequent oxidation of ascorbate.

INTRODUCTION

Hematoporphyrin derivative (HPD) is used in the treatment of malignant disease. The use of Photofrin II, a commercial preparation of HPD with a high proportion of the active di-hematoporphyrin ether (DHE) [10], is considered to be of therapeutic importance. HPD is selectively retained by malignant tumors [15,16] and exposure to red light (630 nm) results in the photodynamic destruction of the tumor tissue. Cytotoxicity has been attributed to singlet oxygen [23,31], an excited state of molecular oxygen and to free radicals [18]. In vitro studies have demonstrated the production of free radicals by hematoporphyrin [3,6,8,11,25], in addition to the well known production of singlet oxygen [8,31]. Lee See et al. [21] have provided clear evidence that the cytotoxic agent of HPD photosensitization is derived from oxygen.

Photodynamic therapy with HPD (DHE) is being investigated as a non-surgical therapeutic option in the treatment of eye tumors [13,14,27—29]. Oxygen-centered free radicals have been shown to mediate an intense uveal and retinal inflammation [29,30] in the eye. We report here light-induced

0304-3835/85/$03.30
Published and Printed in Ireland

generation of hydrogen peroxide and hydroxyl free radical by Photofrin II in the presence of ascorbate. These observations, in conjunction with the fact that the levels of vitamin C in the lens and aqueous humor of the eye are 1.0 ± 0.2 mM [30], suggest that these active oxygen species should be considered when designing a treatment regimen with HPD for tissues having high levels of ascorbate.

MATERIALS AND METHODS

Photofrin II, i.e. a purified preparation of hematoporphyrin derivative with a high proportion of DHE, was purchased from Oncology Research and Development, Inc., Cheektowaga, NY, and was used as received. Sodium ascorbate, catalase, superoxide dismutase and bovine serum albumin were products of Sigma Chemical Co., St. Louis, MO, and were used without further purification. The spin trapping agent, 5,5-dimethylpyrroline-*N*-oxide (DMPO), was a product of Aldrich Chemical Co., Milwaukee, WI. The DMPO was purified as in Ref. 7 and stored at 4°C as an aqueous solution. The concentration of the stock solution was determined using $\epsilon_{232} = 7700\ M^{-1}\ cm^{-1}$ in ethanol [17]. All solutions were prepared in 50 mM phosphate buffer (pH 7). In all experiments the Photofrin II was diluted such that its final concentration produced an absorbance of 2.0 at 365 nm, 1 cm path. Ascorbate measurements were performed at 1 mM. Ascorbate was prepared as a 0.2 M stock solution immediately before use in glass volumetric-ware with deionized water. No loss of reduced ascorbate was noted during the course of a given set of experiments.

Electron spin resonance (ESR) spectra were obtained with a Varian E-4 ESR spectrometer using an aqueous sample cell. Oxygen uptake was monitored with a Yellow Springs Instrument Co., Inc., Model 53 Biological Oxygen Monitor. A slide projector equipped with a 3200 K tungsten bulb was employed as a white light source. The light incident on the sample was filtered through an Oriel IR blocking filter (no. 5205) and an Oriel long pass filter (no. 5130, 50% transmission cut at 530 nm). Using a Yellow Springs Instrument Model 65A radiometer and a Model 6551 probe, the filtered light intensity was determined to be $480\ J\ m^{-2}\ s^{-1}$ for the oxygen uptake experiments and $100\ J\ m^{-1}\ s^{-1}$ for the electron spin resonance experiments, assuming that the cavity grid transmits 50% of the incident light.

RESULTS

Oxygen uptake

Illumination of the HPD-ascorbate solution resulted in consumption of oxygen (see Fig. 1). Control experiments in which either HPD or ascorbate were omitted from the irradiated solution resulted in only a minimal loss of oxygen. Cessation of illumination of the HPD-ascorbate solution halted the rapid oxygen consumption. Addition of 300 units/ml of catalase to the

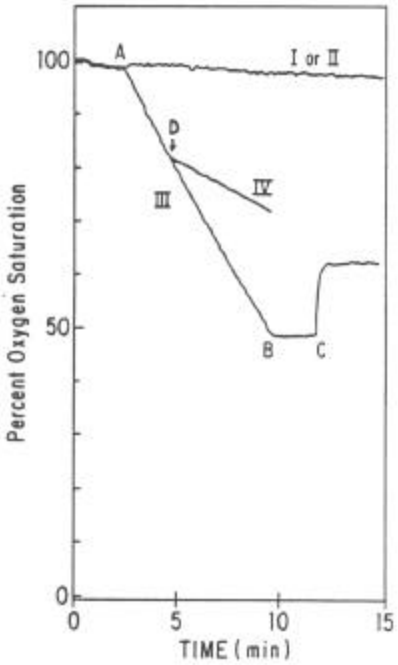

Fig. 1. Oxygen consumption. The irradiation of the solutions began at point A. Irradiation ceased at point B and catalase at 300 units/ml was added at point C. Curves I and II were essentially identical. Solution I contained 1 mM sodium ascorbate in 50 mM phosphate buffer (pH 7.0), while solution II contained Photofrin II in phosphate buffer (pH 7.0). Solution III contained 1 mM ascorbate and Photofrin II in phosphate buffer (pH 7.0). Solution IV was identical to solution III except sodium azide was added to point D, a final concentration of 10 mM. All solutions were air saturated.

illuminated HPD-ascorbate solution initiated the return of 25% of the O_2 that had been consumed ($2H_2O_2$ (catalase) $\rightarrow$ $2H_2O + O_2$). Thus, the return of oxygen to the system with the introduction of catalase indicates the presence of H_2O_2 in the solution. The regeneration of 25% of the oxygen suggests that 50% of the oxygen consumed had been converted to H_2O_2. After the air-saturated HPD-ascorbate solution had been irradiated until only 20% of the oxygen remained, less than 5% of the characteristic absorbance of DHE at 365 nm [24] had disappeared. This represents a loss of less than 1 μmol DHE per 200 μmol O_2. Monitoring the reduction in the ascorbate absorbance at 265 nm showed that it had indeed been oxidized as a result of the irradiation. The addition of azide at 2, 5 and 10 mM decreased the rate of oxygen consumption.

Ascorbate free radical

In a 1 mM aqueous sodium ascorbate solution (pH = 7.0, pK_a of AH_2 = 4.2 [22]) there exists, in equilibrium with AH^- an A, an ESR-detectable con-

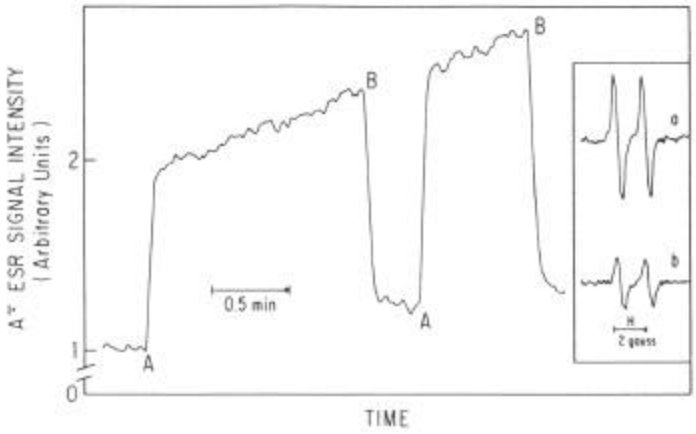

Fig. 2. Ascorbate free radical ESR signal intensity. A solution of 1 mM ascorbate and Photofrin II in 50 mM (pH 7.0) phosphate buffer is irradiated in the ESR cavity. The field has been fixed to the low field peak of the ascorbate doublet. The light was turned on at points A and turned off at points B. The inset shows the ascorbate doublet signal before, a, and during, b, irradiation. The signal is overmodulated, thus no further hyperfine splittings are resolved. Spectrometer settings: 10 mW power, 1 s time constant, 1.25×10^5 gain, 0.5 G modulation amplitude; for the inset the gain was 1.25×10^4 with a scan rate of 25 G/min. All other settings were the same.

centration of $A^{\cdot -}$ [12,22]. (AH_2 = ascorbic acid, AH^- = ascorbate anion, A = dehydroascorbic, and $A^{\cdot -}$ = ascorbate free radical, $pK_a = -0.45$ [20].) The intensity of the ESR signal of $A^{\cdot -}$ was not affected by illumination of a buffered ascorbate solution with filtered white light.

However, when the air-saturated HPD-ascorbate solution was illuminated, an immediate increase in the intensity of the ESR signal of the ascorbate free radical was observed at the start of illumination (a factor of two to three in signal height, see Fig. 2), while cessation of illumination resulted in an immediate decrease in the ascorbate free radical ESR signal intensity.

$$AH^- \xrightarrow{(HPD + h\nu)} A^{\cdot -} \qquad (1)$$

Continued illumination resulted in an additional increase in the intensity of the ESR signal of $A^{\cdot -}$. This is due to the depletion of oxygen from the solution, resulting in a narrowing of the lines with a subsequent increase in height [1]. N_2-purged solutions also produced the immediate increase in signal height at the start of illumination but the additional increase was not observed.

Spin trapping

The $^{\cdot}OH$ free radical is very short-lived because of its extreme reactivity.

We have employed the spin trapping technique [4,19] in which short-lived radicals react with a spin trap producing a much longer-lived free radical spin adduct. The spin adduct will accumulate to a concentration sufficient to be observed by ESR.

$$DMPO + R^{\cdot} \rightarrow DMPO/R \quad (2)$$

The inclusion of the spin trap DMPO at 50 mM in an illuminated HPD-ascorbate solution resulted in the detection of a spin adduct with hyperfine splitting constants of DMPO/OH (see Fig. 3) ($a_N = a_H = 15.0$ G [4]). The inclusion of Fe(III)EDTA at catalytic levels, 1 μM, resulted in a significant increase in the DMPO/OH spin adduct signal intensity. When 0.1 M ethanol was included in the HPD-ascorbate-Fe(III)EDTA spin-trapping solution the spectrum of the DMPO/α-hydroxyethyl radical adduct ($a_N = 15.8$ G and $a_H = 23.0$ G [4]) was observed. The observation of this carbon-centered radical adduct is consistent with the following mechanism:

$$^{\cdot}OH + CH_3CH_2OH \rightarrow H_2O + CH_3\dot{C}HOH \quad (3)$$

$$DMPO + CH_3C\dot{H}OH \rightarrow DMPO/CH_3\dot{C}HOH \quad (4)$$

When sodium formate was substituted for ethyl alcohol, an ESR signal previously attributed to the formate radical spin adduct of DMPO was observed ($a_N = 15.8$ G, $a_H = 18.6$ G [4]).

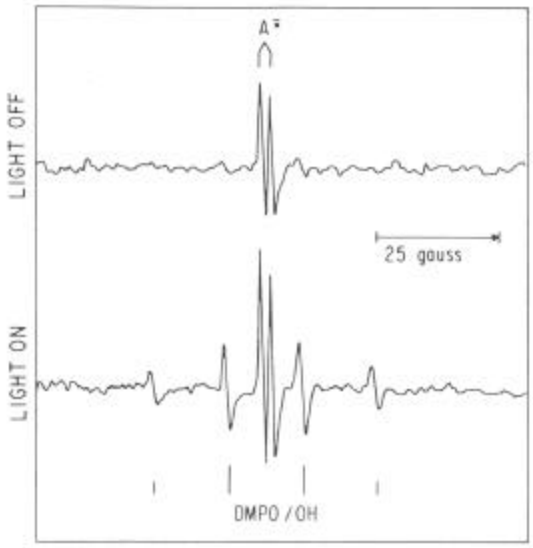

Fig. 3. Spin trapping of $^{\cdot}OH$. A solution of 1 mM ascorbate, 1 μM Fe(III) EDTA and Photofrin II with 50 mM DMPO is irradiated in the ESR cavity. Spectrometer settings were as in Fig. 2.

These observations are consistent with the formation of the $^{\cdot}OH$ free radical by this photosensitizing system. Catalase was able to slow the rate at which the DMPO/OH spin adduct appeared, demonstrating the role of hydrogen peroxide in the formation of $^{\cdot}OH$. Ascorbate reduces Fe(III)EDTA to Fe(II)EDTA which then reacts with H_2O_2 to form $^{\cdot}OH$ via the Fenton reaction.

$$Fe(III)EDTA + AH^- \rightarrow A^{\cdot-} + Fe(II)EDTA + H^+ \quad (5)$$

$$Fe(II)EDTA + H_2O_2 \rightarrow {}^{\cdot}OH + OH^- + .Fe(III)EDTA \quad (6)$$

CONCLUSIONS

A possible mechanism for the production of H_2O_2 in this system is:

$$HPD(S_o) + h\nu \rightarrow HPD(S^*) \rightarrow HPD(T^*) \quad (7)$$

$$HPD(T^*) + AH^- \rightarrow HPD^{\cdot-} + A^{\cdot-} \quad (8)$$

$$HPD^{\cdot-} + O_2 \rightarrow O_2^{\cdot-}\ (HO_2^{\cdot}) + HPD(S_o) \quad (9)$$

$$HPD(T^*) + {}^3O_2 \rightarrow {}^1O_2 + HPD(S_o) \quad (10)$$

$${}^1O_2 + AH^- \rightarrow O_2^{\cdot-}/HO_2^{\cdot} + A^{\cdot-} \quad (11)$$

$$O_2^{\cdot-}/HO_2^{\cdot} + A^{\cdot-} \rightarrow H_2O_2 + A \quad (12)$$

where S_o, S* and T* are the ground state singlet, excited singlet and excited triplet states of HPD, respectively.

Reactions 8 and 9 are in accord with the observation of the reduced hematoporphyrin radical in Refs. 8, 11, 25, however this species has not yet been observed for DHE. The decrease in oxygen uptake with the introduction of azide ion suggests a role for singlet oxygen. The rate constant for the quenching of $^1O_2$ by azide ion is $2.2 \times 10^8\ M^{-1}\ s^{-1}$. Ascorbate has been found to be an effective quencher of singlet oxygen, $k = 8 \times 10^6\ M^{-1}\ s^{-1}$ [2,9,26]. Chou and Khan [9] have provided evidence that ascorbate chemically quenches singlet oxygen, thus reactions 10 and 11 may be the principal mechanism by which $A^{\cdot-}$ and superoxide could be formed. H_2O_2 would result from the production of superoxide by this photodynamic system. Addition of superoxide dismutase at 150 units ml^{-1} did not affect the rate of oxygen consumption. The DMPO-superoxide spin adduct could not be detected in the spin-trapping experiments. However, it is extremely difficult to detect $O_2^{\cdot-}/HO_2^{\cdot}$ in a system containing 1 mM AH^- as well as substantial $A^{\cdot-}$ by the techniques employed in these experiments [5].

The oxidation of ascorbate, and the production of H_2O_2 and $^{\cdot}OH$ by DHE

and light needs to be considered when dealing with tissue having high levels of ascorbate, such as the eye. In addition O_2^- and H_2O_2 have been implicated in cataract formation [30]. The lens has a low level of catalase activity [32] and thereby may be vulnerable to a high flux of H_2O_2. Thus appropriate eye protection should be provided for patients who have received HPD until its concentration in the eye has declined to a safe level.

ACKNOWLEDGEMENTS

We gratefully acknowledge NSF equipment grants no. PRM-8109079 and no. TFI 8109273 as well as the support of the Research Corporation and the Haines Fund. GRB was a recipient of an NIH-NRSA award, no. 3 F33 ESO5285-O1S1.

REFERENCES

1 Backer, J.M., Budker, V.G., Eremenko, S.I. and Molin, Y.N. (1977) Detection of the kinetics of biochemical reactions with oxygen using exchange broadening in the ESR spectra of nitroxide radicals. Biochim. Biophys. Acta, 460, 152–156.
2 Bodaness, R.S. and Chan, P.C. (1979) Ascorbic acid as a scavenger of singlet oxygen. FEBS Lett., 105, 195–196.
3 Bonnett, R., Lambert, C., Land, E.J., Sourides, P.A., Sinclair, R.S. and Truscott, T.G. (1983) The triplet and radical species of haematoporphyrin and some of its derivatives. Photochem. Photobiol., 38, 1–8.
4 Buettner, G.R. (1982) The spin trapping of superoxide and hydroxyl free radicals. In: Superoxide Dismutase, Vol. 2, pp. 63–81. Editor: L.W. Oberley. CRC Press, Boca Raton, FL.
5 Buettner, G.R., Doherty, T.P. and Bannister, T.D. (1984) Hydrogen peroxide and hydroxyl radical formation by methylene blue in the presence of ascorbic acid. Radiat. Environ. Biophys., 23, 235–243.
6 Buettner, G.R. and Oberley, L.W. (1980) The apparent production of superoxide and hydroxyl radicals by hematoporphyrin and light as seen by spin-trapping. FEBS Lett., 121, 161–164.
7 Buettner, G.R. and Oberley, L.W. (1978) Considerations in the spin trapping of superoxide and hydroxyl radicals in aqueous systems using 5,5-dimethylpyrroline-1-oxide. Biochem. Biophys. Res. Commun., 83, 69–74.
8 Cannistraro, S. and Van de Vorst, A. (1977) Photosensitization by hematoporphyrin: ESR evidence for free radical induction in unsaturated fatty acids and for singlet oxygen production. Biochem. Biophys. Res. Commun., 74, 1177–1185.
9 Chou, P.-T. and Khan, A.U. (1983) L-Ascorbic acid quenching of singlet delta molecular oxygen in aqueous media: generalized antioxidant property of vitamin C. Biochem. Biophys. Res. Commun., 115, 932–937.
10 Dougherty, T.J., Potter, W.R. and Weishaupt, K.R. (1984) The structure of the active component of hematoporphyrin derivative. In: Porphyrins in Tumor Phototherapy, pp. 23–35. Editors: A. Andreoni and R. Cubeddu. Plenum Press, New York.
11 Felix, C.C., Reszka, K. and Sealy, R.C. (1983) Free radicals from photoreduction of hematoporphyrin in aqueous solution. Photochem. Photobiol., 37, 141–1437.
12 Foerster, G.V., Weiss, W. and Straudinger, H. (1965) Electron spin resonance determination on semidehydroascorbic acid. Ann. Chem., 690, 166–169.
13 Gomer, C.J., Doiron, D.R., Jester, J.V., Szirth, B.C. and Murphree, A.L. (1983)

Hematoporphyrin derivative photoradiation therapy for treatment of intraocular tumors: examination of acute normal ocular tissue toxicity. Cancer Res., 43, 721–727.

14 Gomer, C.J., Doiron, D.R., White, L., Jester, J.V., Dunn, S., Szirth, B.C., Razum, N.J. and Murphree, A.L. (1984) Hematoporphyrin derivative photoradiation induced damage to normal and tumor tissue of the pigmented rabbit eye. Curr. Eye Res., 3, 229–237.

15 Gomer, C.J. and Dougherty, T.J. (1979) Determination of ^{3}H and ^{14}C hematoporphyrin derivative distribution in malignant and normal tissue. Cancer Res., 39, 146–151.

16 Gregorie, H.B., Horger, E.O., Ward, J.L., Green, J.F., Richards, T., Robertson, H.C. and Stevenson, T.B. (1968) Hematoporphyrin-derivative fluorescence in malignant neoplasms. Ann. Surg., 167, 820–828.

17 Hamer, J. and Macaluso, A. (1964) Nitrones. Chem. Rev., 64, 473–495.

18 Hariharan, P.V., Courtney, J. and Elecczko, S. (1980) Production of hydroxyl radicals in cell systems exposed to haematoporphyrin and red light. Int. J. Radiat. Biol., 27, 691–694.

19 Janzen, E.G. (1980) A critical review of spin trapping in biological systems. In: Free Radicals in Biology, Vol. 4, pp. 116–154. Editor: W.A. Pryor. Academic Press, New York.

20 Laroff, G.P., Fessenden, R.W. and Schuler, R.H. (1972) The electron spin resonance spectra of radical intermediates in the oxidation of ascorbic acid and related substances. J. Am. Chem. Soc., 94, 9062–9073.

21 Lee See, K., Forbes, I.J. and Betts, W.H. (1984) Oxygen dependency of photocytotoxicity with haematoporphyrin derivative. Photochem. Photobiol., 39, 631–634.

22 Lewin, S. (1976) Vitamin C: Its Molecular Biology and Medical Potential. Academic Press, London.

23 Moan, J., Petersen, E.O. and Christensen, T. (1979) The mechanism of photodynamic inactivation of human cells in vitro in the presence of haematoporphyrin. Br. J. Cancer, 39, 398–407.

24 Poletti, A., Murgia, S.M., Pasqua, A., Reddi, E. and Jori, G. (1984) Photophysical and photosensitizing properties of Photofrin II. In: Porphyrins in Tumor Phototherapy, pp. 37–43. Editors: A. Andreoni and R. Cubeddu. Plenum Press, New York.

25 Reszka, K. and Sealy, R.C. (1984) Photooxidation of 3,4-dihydroxyphenylalanine by hematoporphyrin in aqueous solutions: an electron spin resonance study using 2,2,6,6-tetramethyl-4-piperidone-1-oxyl(tempone). Photochem. Photobiol., 39, 293–299.

26 Rooney, M.L. (1983) Ascorbic acid as a photooxidation inhibitor. Photochem. Photobiol., 38, 619–621.

27 Sery, T.W. (1979) Photodynamic killing of retinoblastoma cells with hematoporphyrin and light. Cancer Res., 39, 96–100.

28 Sery, T.W. and Doughtery, T.J. (1984) Photoradiation of rabbit ocular malignant melanoma sensitized with hematoporphyrin derivative. Curr. Eye Res., 3, 519–528.

29 Sery, T.W. and Petrillo, R. (1984) Superoxide anion radical as an indirect mediator in ocular inflammatory disease. Curr. Eye Res., 3, 243–252.

30 Varma, S.D., Chand, D., Sharma, Y.R., Kuck, J.F. and Richards, R.D. (1984) Oxidative stress on lens cataract formation: role of light and oxygen. Curr. Eye Res., 3, 35–57.

31 Weishaupt, K.R., Gomer, C.J. and Dougherty, T.J. (1976) Identification of singlet oxygen as the cytotoxic agent in photoinactivation of a murine tumor. Cancer Res., 36, 2326–2329.

32 Zeller, M.A. (1953) Contributions to the enzymology of the normal and cataractous lens III. On the catalase of the cystalline lens. Am. J. Ophthalmol. 36, 51–53.