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

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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 nonsurgical 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
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 $1.0 \pm 0.2$ 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 $\epsilon_{252} = 7700$ 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
illuminated HPD-ascorbate solution initiated the return of 25% of the O₂ that had been consumed (2H₂O₂ (catalase) → 2H₂O + O₂). Thus, the return of oxygen to the system with the introduction of catalase indicates the presence of H₂O₂ in the solution. The regeneration of 25% of the oxygen suggests that 50% of the oxygen consumed had been converted to H₂O₂. 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 O₂. 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, pKₐ of AH₂ = 4.2 [22]) there exists, in equilibrium with AH⁻ an Λ, an ESR-detectable con-
Fig. 2. Ascorbate free radical ESR signal intensity. A solution of 1 mM ascorbate and Photosin 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, $1.25 \times 10^4$ gain, 0.5 G modulation amplitude; for the inset the gain was $1.25 \times 10^4$ with a scan rate of 25 G/min. All other settings were the same.

centration of $A^{-}$ [12,22]. ($AH_2$ = ascorbic acid, $AH^{-}$ = ascorbate anion, $A$ = dehydroascorbic, and $A^{-}$ = ascorbate free radical, $pK_a = -0.45$ [20].) The intensity of the ESR signal of $A^{-}$ 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.

\[
AH^{-} (\text{HPD} + h\nu) \rightarrow A^{-} \quad (1)
\]

Continued illumination resulted in an additional increase in the intensity of the ESR signal of $A^{-}$. 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.

\[ \text{DMPO} + \cdot \text{R}^* \rightarrow \text{DMPO/R} \]  

(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) \( (a_N = a_H = 15.0 \text{ G} [4]) \). The inclusion of Fe(III)EDTA at catalytic levels, 1 \( \mu \text{M} \), 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/\( \alpha \)-hydroxyethyl radical adduct \( (g_N = 15.8 \text{ G} \text{ and } a_H = 23.0 \text{ G} [4]) \) was observed. The observation of this carbon-centered radical adduct is consistent with the following mechanism:

\[ \cdot \text{OH} + \text{CH}_3\text{CH}_2\text{OH} \rightarrow \text{H}_2\text{O} + \text{CH}_3\cdot\text{CHOH} \]  

(3)

\[ \text{DMPO} + \text{CH}_3\cdot\text{CHOH} \rightarrow \text{DMPO}/\text{CH}_3\cdot\text{CHOH} \]  

(4)

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

Fig. 3. Spin trapping of \( \cdot \text{OH} \). A solution of 1 mM ascorbate, 1 \( \mu \text{M} \) 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 \( \cdot \text{OH} \) free radical by this photosensitizing system. Catalase was able to slow the rate at which the DMPO/\( \cdot \text{OH} \) spin adduct appeared, demonstrating the role of hydrogen peroxide in the formation of \( \cdot \text{OH} \). Ascorbate reduces Fe(III)EDTA to Fe(II)EDTA which then reacts with \( \text{H}_2\text{O}_2 \) to form \( \cdot \text{OH} \) via the Fenton reaction.

\[
\text{Fe(III)EDTA} + \text{AH}^- \rightarrow \cdot \text{A}^- + \text{Fe(II)EDTA} + \text{H}^+ \\
\text{Fe(II)EDTA} + \text{H}_2\text{O}_2 \rightarrow \cdot \text{OH} + \text{OH}^- + \text{Fe(III)EDTA}
\]

CONCLUSIONS

A possible mechanism for the production of \( \text{H}_2\text{O}_2 \) in this system is:

\[
\text{HPD}(S_0) + \text{hv} \rightarrow \text{HPD}(S^*) \rightarrow \text{HPD(T*)} \\
\text{HPD(T*)} + \text{AH}^- \rightarrow \text{HPD}^- + \cdot \text{A}^- \\
\text{HPD}^- + \text{O}_2 \rightarrow \cdot \text{O}_2^- + (\text{HO}_2^-) + \text{HPD}(S_0) \\
\text{HPD(T*)} + \cdot \text{O}_2 \rightarrow \cdot \text{O}_2^- + \text{HPD}(S_0) \\
\cdot \text{O}_2 + \text{AH}^- \rightarrow \cdot \text{O}_2^-/\text{HO}_2^- + \cdot \text{A}^- \\
\cdot \text{O}_2^-/\text{HO}_2^- + \cdot \text{A}^- \rightarrow \text{H}_2\text{O}_2 + \cdot \text{A}
\]

where \( S_0, S^* \) 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 \( \cdot \text{O}_2 \) by azide ion is \( 2.2 \times 10^8 \text{M}^{-1} \text{s}^{-1} \). Ascorbate has been found to be an effective quencher of singlet oxygen, \( k = 8 \times 10^8 \text{M}^{-1} \text{s}^{-1} \) \([2,9,26]\). 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 \( \cdot \text{A}^- \) and superoxide could be formed. \( \text{H}_2\text{O}_2 \) 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 \( \cdot \text{O}_2^-/\text{HO}_2^- \) in a system containing 1 mM \( \text{AH}^- \) as well as substantial \( \cdot \text{A}^- \) by the techniques employed in these experiments \([5]\).

The oxidation of ascorbate, and the production of \( \text{H}_2\text{O}_2 \) and \( \cdot \text{OH} \) by DHE
and light needs to be considered when dealing with tissue having high levels of ascorbate, such as the eye. In addition O$_3$ and H$_2$O$_2$ 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$_2$O$_2$. 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.

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