PHOTOSENSITIZATION BY HEMATOPORPHYRIN: ESR EVIDENCE FOR FREE RADICAL INDUCTION IN UNSATURATED FATTY ACIDS AND FOR SINGLET OXYGEN PRODUCTION

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SUMMARY

Hematoporphyrin ability to photoreact by type I and type II mechanisms was investigated in some model systems. At room temperature, visible irradiation of hematoporphyrin-unsaturated fatty acids and hematoporphyrin-cholesterol systems resulted in the Electron Spin Resonance (ESR) spectrum of the hematoporphyrin free radical. Triplet state hematoporphyrin is shown to be involved in the electron transfer from the lipid moiety. Moreover an ESR method to monitor the singlet oxygen production by hematoporphyrin was used. β-carotene effect on both mechanisms (type I and type II) was tested.

INTRODUCTION

It is generally accepted that photohemolysis of erythrocytes associated with the porphyrias results from damage to cells membranes, following photoexcitation of porphyrin molecules in the presence of oxygen (1-6). Photooxidation of membrane proteins and/or photoperoxidation of unsaturated fatty acids (UFA) of membrane lipids are believed to be responsible for such a damage (3-11). Very little is known about the primary photochemical events leading to the photosensitization, even if a photodynamic process of some kind is usually postulated.

In the case of erythropoietic protoporphyria, Goldstein

Abbreviations: ESR, Electron Spin Resonance; UFA, Unsaturated Fatty Acids; HP, Hematoporphyrin; $^1O_2$, singlet oxygen.

and Harber (8) have postulated that excitation of protoporphyrin results in an oxygen-dependent electron transfer to double bond of cells membrane producing lipid peroxidation. Indeed, porphyrins semm to be able to interact by a type I mechanism in the presence of suitable substrates (6,12-14). In the other side, β-carotene, known as an extremely effective singlet oxygen quencher ($^1O_2$) (15), has been shown to prevent, almost completely the photosensitizing action of porphyrins *in vitro* (4, 16) and *in vivo* (17,18). This strongly suggested that $^1O_2$ could be the primary oxidation agent leading to cell membrane damage. Intermediacy of $^1O_2$ has been postulated in the oxidation of UFA (4,7), cholesterol (19) and certain amino acids(4,7) in the photosensitization of red cells by porphyrins.

In a recent study on the photohemolysis of erythrocytes induced by hematoporphyrin (HP), Nilsson *et al.*(20) suggest that both mechanisms, type I and type II, are probably involved. HP is adsorbed to red cells surface, hence type I reaction would be favoured by the more direct interaction between sensitizer molecules and acceptors on the membrane. Along this line and taking into account the relative lipid solubility of HP, one can assume that its local environment is lipid; thus mixtures HP-UFA would constitute a suitable model to study an eventual direct interaction occurring during photosensitization of red cells by HP.

We report here the ESR results obtained from HP-UFA and HP-cholesterol systems, under visible irradiation at room temperature. In all cases, the photoreduced HP free radical was observed. Moreover hematoporphyrin reaction by type II mechanism in various solvents is demonstrated by an ESR method of detecting $^1O_2$. β-carotene effect on both mechanisms was tested.

MATERIALS AND METHODS

Oleic, linoleic and linolenic acids, linoleic acid methyl ester and all trans β-carotene were Sigma products. Cholesterol acetate was from Calbiochem, hematoporphyrin IX dihydrochloride from Fluka. Methanol, ethanol and chloroform, all for spectroscopic use, were purchased from Merck. 2,2,6,6-tetramethyl-piperidin was an Aldrich Europa product.

The light source for all irradiation experiments was a 500 watt high pressure mercury lamp(Osram). Cut-off filters (Jenear Glaswork Schott) were used to select the opportune wavelengths. ESR spectra were recorded at 25°C, by a Varian spectrometer E-3 type, with 100 kHz modulation and equipped with a variable temperature accessory. All g-factor measurements were performed by graphical interpolation between the two centermost lines of Mg^{++}.

To promote solubility of HP in UFA, 10 ml of hematoporphyrin 5.10^{-5}M in chloroform were added to one ml of UFA (all UFA studied were liquid); then purified nitrogen was bubbled through until all chloroform was evaporated. Thus, HP resulted dissolved in UFA at an approximate concentration of 5.10^{-4}M. HP-cholesterol solutions were prepared in chloroform; solutes were concentrated up respectively up to 4.10^{-5}M and 1 M by bubbling with N_2. To perform experiments in deareated conditions, aliquots of solutions were placed in 3 mm ID quartz tubes and sealed after five cycles of freezing-thawing under a vacuum of 10^{-5} Torr. Samples were irradiated in the cavity of the spectrometer through a collimating lens at 15 cm by the lamp.

Singlet oxygen production by HP was demonstrated by the ESR method set up by Lion et al (21). HP was dissolved in different organic solvents at a concentration of 10^{-5}M; solutions were then bubbled with O_2 for 15 min. (to avoid evaporation, O_2 was bubbled through the solvent before passing it over the sample). Finally 2,2,6,6-tetramethyl-piperidin was added at a concentration of $1.15.10^{-3}$M. A volume of 1.5 ml of such a solution was irradiated in a thermostatic quartz cuvette (18 ± 1°C) under one atmosphere of air. Aliquots were taken in calibrated capillaries (Drummond microcaps, 50µl) before exposure to light and after different exposure times. ESR quantitative measurements were performed by comparison with a calibrated concentration of nitroxide in solution.

RESULTS AND DISCUSSION

Irradiation at room temperature of degassed HP-UFA systems and chloroform solutions of HP-cholesterol with light (λ>340nm) resulted in the ESR spectrum shown in Fig. 1a. When the light was turned off the signal decayed very quickly (<1 sec) and we failed to obtain a higher resolution by lowering the modulation amplitude. It consists in a single line whose g-value and peak-to-peak width slightly varied with the different systems studied.

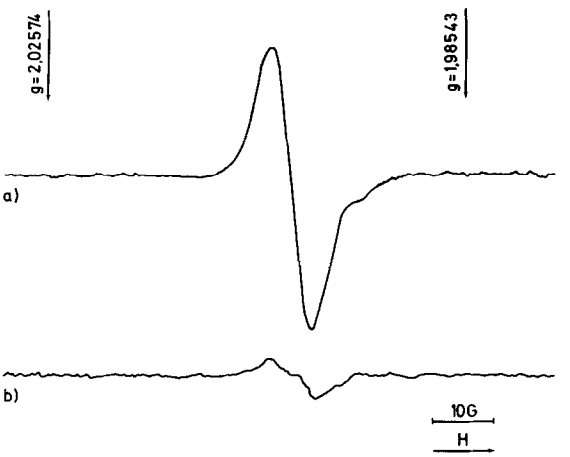

Fig. 1. a) ESR spectrum of deareated HP-UFA and HP-cholesterol systems;
b) same as (a) but in the presence of β-carotene (10^{-4}M). Irradiation in the cavity, with λ>340 nm at 25°C. Microwave power level: 10 mW. Modulation amplitude: 10 Gauss.

Table 1. g-values and line width of the ESR signal obtained in the photoreaction of hematoporphyrin with different unsaturated fatty acids and cholesterol.

System	g-value	ΔH (Gauss)
HP + Oleic acid	2.0020±0.0002	5.5±0.5
HP + Linoleic acid	2.0022±0.0001	5.7±0.3
HP + Linolenic acid	2.0024±0.0002	5.9±0.5
HP + Linoleic ac. methyl ester	2.0021±0.0001	5.7±0.3
HP + Cholesterol (in chloroform)	2.0026±0.0002	6.2±0.5

These values, which have been resumed in Table 1, are in excellent agreement with those found for free porphyrin radical by many authors (12-14), who investigated the photoreduction of several porphyrins (HP included), in the presence of various reducing

agents. In the present case, we can affirm that photoexcitation of HP results in an electron transfer from the lipid moiety, to form the photoreduced hematoporphyrin free radical. Since chloroform solution of HP alone did not show any detectable signal, it can be inferred that the same electron transfer process occurs in the HP-cholesterol system.

In the presence of air, or O_2, no signal could be detected. This was also the case when β-carotene (10^{-4}M) had been previouly added to deareated solutions, as it is shown in Fig. 1b.

Singlet as well as triplet state of porphyrins have been postulated as the excited states responsible for their photoreduction (12,14). However in the present case, direct involvement of singlet state is ruled out by the fact that no oxygen-effect was encountered on the HP fluorescence emission, in agreement with Cauzzo et al. (22), while O_2 drastically reduced the ESR signal. In addition, since β-carotene quenches triplet state of chlorophyll (23) and of other photosensitizers (24), the inhibitory effect on the photoinduced electron transfer indicates the participation of triplet state of HP.

The above observations are consistent with the following scheme:

$$ {}^1HP \xrightarrow{h\nu} {}^1HP^* \xrightarrow[\text{crossing}]{\text{intersystem}} {}^3HP^* $$

$$ {}^3HP^* + L \longrightarrow {}^1HP^{\cdot-} + L^{\cdot+} $$

Where $^1HP^*$ and $^3HP^*$ indicate respectively the singlet and triplet excited states of HP, while L represents UFA or cholesterol. Radical $L^{\cdot+}$ escaped detection, probably owing to fast recombination.

No correlation was found between the amount of HP free radicals and the number of unsaturated double bonds of UFA, when they were compared at the same molarity.

Among the functional groups of the UFA theoretically most susceptible to a photoreducing process are the OH groups; but the result obtained with linoleic acid methyl ester, where OH is replaced by the methyl group, rules out the intermediacy of the OH groups in the photoreduction of HP. Thus at present we are not able to predict the sites where the free radicals are induced in UFA or in cholesterol correspondingly to the photoreduction of HP. Anyway the most important conclusion emerging from these results is that free radicals are photoinduced in HP-UFA and HP-cholesterol systems; these very reactive species could constitute the initiators of the radicalar chain reaction leading to lipid peroxidation and consequently to membrane damage.

The method used to monitor the production of singlet oxygen by HP is based on the high specificity of the tertiary amine 2,2,6,6-tetramethyl-piperidin for $^1O_2$ and the stabilization of the oxydation products under the form of nitroxide free radicals easily detectable by ESR (21). In other words, determination of nitroxide radical concentration induced after irradiation of HP in the presence of O_2 and the amine, should bear a direct relationship to $^1O_2$ produced via triplet HP.

Experiments were performed in three different solvents and samples were irradiated with wavelengths longer than 340 nm. The rate of nitroxide radicals formation is reported in Fig. 2. Prior to irradiation a slight ESR signal was already observed. This spectrum characterizes nitroxide free radicals present as an impurity in the commercial product: its concentration does

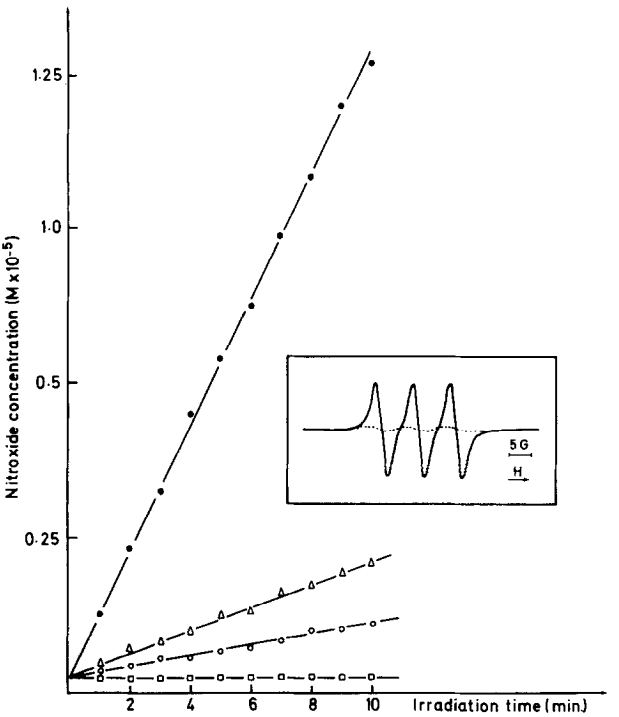

Fig. 2. Nitroxide concentration versus light exposure duration
HP (10^{-5}M) dissolved in :
● chloroform
▲ ethanol
○ methanol
□ chloroform + β-carotene.
2,2,6,6 tetramethyl-piperidin concentration: $1.15.10^{-3}$M.
Insert: ESR spectra before (....) and after (——) illumination (λ>340 nm). Microwave power level: 2 mW. Modulation amplitude: 2 Gauss. Temperature:25°C.

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not exceed 0.3 %. HP dissolved in chloroform shows a higher rate of $^1O_2$ as compared to HP dissolved in ethanol or in methanol. This could be due to the higher O_2-solubility in chloroform (25) and to a solvent effect on the rate decay of $^1O_2$ and/or on the rate constant for the reaction of $^1O_2$ with the amine (26).

Adding β-carotene to the samples, at a concentration of 10^{-5}M in chloroform resulted in the complete quenching of $^1O_2$, as it is shown in Fig. 2. It should be noted that the β-caro-

tene quenching effect was encountered when irradiation was carried out in the wavelengths range where it absorbs (λ>340 nm) as well as where it does not (λ>550 nm). In addition β-carotene is quickly destroyed during irradiation in the presence of HP, as resulted by monitoring its optical absorption at 460 nm. In our conditions, 15 min irradiation (λ> 340 nm) resulted in more than 50 % optical bleaching of β-carotene (10^{-5}M). This is in agreement with Anderson et al. (27) who found a considerable destruction of β-carotene in chloroform-ethanol mixture due to singlet oxygen generated either by photosensitization of toluidene blue or by radiofrequency discharge.

In conclusion, the above experimental results prove unambigously the capacity of HP to interact by a radicalar mechanism within the lipid milieu, and they provide a direct information about the singlet oxygen producing ability of HP.

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