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Photodynamic Reactions in Dimyristoyl-L-α-phosphatidylcholine (DMPC) Liposomes

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The photodynamic reactions of singlet oxygen in the presence of sensitizers (methylene blue, eosin, porphyrin) and substrates (9,10-dimethylantracene, anthroyl stearic acid, diphenylfurane) in dimyristoyl-L-α-phosphatidylcholine (DMPC) liposomes have been studied. Evidence of the involvement of singlet oxygen and its diffusion through the phospholipid bilayer has been achieved. Possible correlations with erythopoietic porphyrias are discussed.

Introduction

Erythropoietic porphyrias, a human disease genetically governed, is associated with or characterized by abnormalities in heme biosynthesis that induce the concentration of heme precursors and tetrapyrrol compounds in the cytoplasm of the red cell [1]. Clinical manifestations are mainly the increase in the fragility of the red cell membrane and consequent splenic phagocytosis with a marked decrease in the cell survival time, and skin photosensitivity manifested by the formation of injuries following exposure to natural light.

It is generally accepted that in pathological processes such as carcinogenesis, in aging and in general degradative processes of the cell the singlet oxygen is involved [2–6]. Moreover singlet oxygen was proposed to be the direct initiator of lipid peroxidation by reacting with the diene bonds of unsaturated lipids, giving rise to lipid hydroperoxides [7, 8].

Because of their nature, we can suppose that porphyrias act as dye sensitizers for singlet oxygen produced by light or by cellular biochemical pathways. Singlet oxygen could diffuse inside the lipid bilayer starting the degradative photooxidation processes of membrane components [9, 10]. In order to prove this photodynamic effect we studied some model systems.

As membrane model we choose dimyristoyl-L-α-phosphatidylcholine (DMPC) vesicles (liposomes). We simulated in the liposomes the hypothetical photochemical situation of the pathological red blood cells simply by trapping in them a photosensitizer for singlet oxygen and a substrate. Control sensitizers, methylene blue and eosin in parallel with the complex hemoglobin–porphyrin were used; substrates for the photodynamic reaction were 9,10-dimethylanthracene, anthroyl stearic acid and diphenylfurane for eosin, emoglobin and porphyrin complex.

Experimental

Materials

9,10-dimethylanthracene, methylene blue, eosin, hemoglobin, protoporphyrin IX, dimyristoyl-L-α-phosphatidylcholine were purchased from Sigma; diphenylfurane from Eastman Organic Chemical, and anthroyl stearic acid was a kind gift of Prof. G. Settimi. All products were used without further purification.

Preparation of the sensitizer-substrate containing liposomes

Solutions of sensitizers (10^{-6} to 10^{-4} *M* in 0.1 *M* phosphate buffer, pH 7.4, in H_2O or D_2O), of DMPC (80 mg/ml in $CHCl_3$), and singlet oxygen substrates (10^{-3} *M* in $CHCl_3$) were prepared. A volume of substrate solution corresponding to 10^{-7} mol and a volume of DMPC corresponding to 10^{-5} mol were added. The mixture was dried in a rotary evaporator under a nitrogen stream and resuspended in 5 ml of the sensitizer buffered solution. The suspension was then sonicated at about 4 °C under a flux of nitrogen for 10 min at 70 W with a Branson sonifier. The excess of sensitizer was removed

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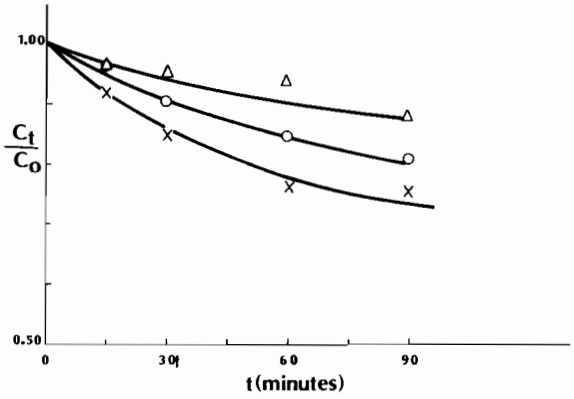

Fig. 1. × 9,10-dimethylanthracene 10^{-2} *M* in DMPC liposomes; ○ 9,10-dimethylanthracene 10^{-2} *M* in DMPC liposomes + α-tocopherol 1.5 × 10^{-3} *M*, ▲ 9,10-dimethylanthracene 10^{-2} *M* in DMPC liposomes + α-tocopherol 1.5 × 10^{-2} *M*.

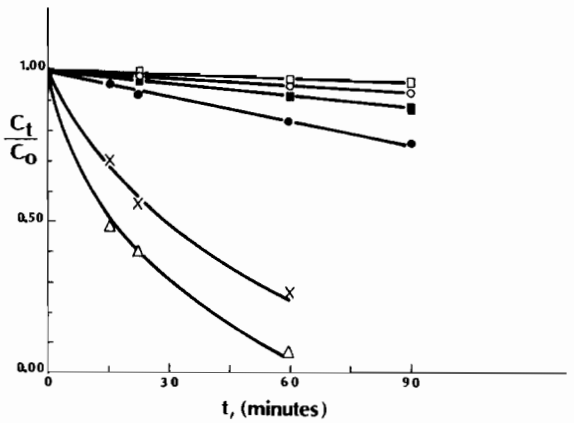

Fig. 2 DMPC liposomes with methylene blue (MB). Substrates 10^{-2} *M* in DMPC liposomes. □ MB 10^{-4} *M* in H_2O buffer; anthroyl stearic acid, ■ MB 10^{-4} *M* in D_2O buffer, anthroyl stearic acid, ○ MB 10^{-5} *M* in H_2O buffer, 9,10-dimethylanthracene, ● MB 10^{-5} *M* in D_2O buffer, 9,10-dimethylanthracene; X MB 10^{-4} *M* in H_2O buffer, diphenylfurane; + MB 10^{-4} *M* in D_2O buffer, diphenylfurane.

through a Sephadex G 15 column, equilibrated in the phosphate buffer. The liposome fractions were monitored at 255 nm and collected, while the sensitizer pratically did not elute. All operations were carried out at 4 °C. Three ml of the liposome suspension were put in a test tube and irradiated under a stream of water saturated oxygen in an adapted Perkin-Elmer spectrofluorimeter, through a cut-off filter 3-70 (transmittance 10% at 482 nm, 1% at 472 nm). The irradiation was periodically interrupted and the disappearance of the substrate measured by the decrease of its fluorescence intensity.

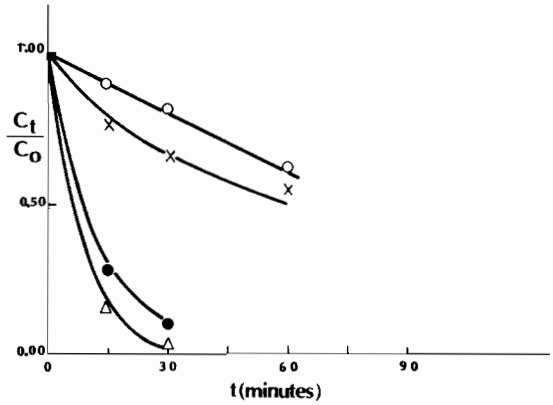

Fig. 3. DMPS liposomes with eosin. Diphenylfurane 10^{-2} *M* in DMPC liposomes. ○ Eosin 10^{-6} *M* in H_2O buffer. × Eosin 10^{-5} *M* in H_2O buffer. + Eosin 10^{-5} *M* in D_2O buffer; △ Eosin 10^{-5} *M* in H_2O buffer.

Plots of $I/I_o = C/C_o = f(t)$ were obtained (where I and C are referred to the intensity and to the concentration of the substrate at t time respectively, and I_o and C_o are the corresponding values at time t = 0).

Preparation of Complex containing Liposomes

Protoporphyrin IX was dissolved in the minimum amount of ethanol and diluted with phosphate buffer to a concentration of 10^{-5} *M*. The complex was prepared by adding to a hemoglobin solution (10^{-4} *M*) the same volume of the protoporphyrin IX solution. The dried DMPC (80 mg) – diphenylfurane (10^{-7} mol) mixture was suspended in 3 ml of the complex solution and sonicated at the same conditions described above. The excess of the complex was removed through a Sephadex G 2000 column, following the procedure previously described. All operations were carried out at 40 °C. The irradiation of the sample was performed as described above.

Results and Discussion

In Fig. 1 are reported the results obtained on the liposomes of DMPC entraping 9,10-dimethylanthracene. In the same figure parallel experiments in the presence of two different concentrations of the antioxidant α-tocopherol are also reported.

A general decrease of C/C_o as a function of time indicates that the photodynamic effect takes place. Moreover a reduction of the reaction is observed in the presence of α-tocopherol. The reduction is function of the antioxidant concentration.

In Fig. 2 are reported results relative to the system DMPC liposomes-methylene blue and three different substrates in H_2O or D_2O buffer. The analogous results

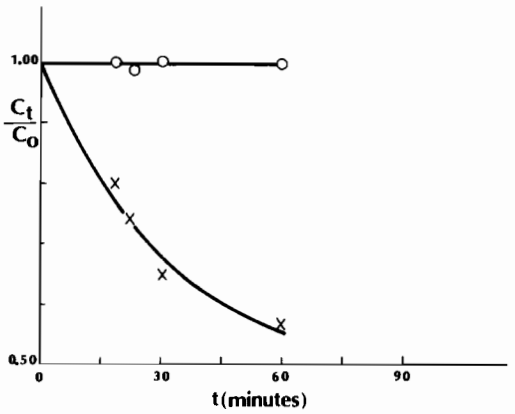

Fig. 4. DMPC liposomes with diphenylfurane. ○ in H_2O buffer; × in H_2O buffer with porphyrin complex.

for the system liposomes-eosin and diphenylfurane as substrate in H_2O or D_2O buffer are reported in Fig. 3. From a close inspection of the results obtained can be derived some general conclusions.

In all the systems studied, it is evident that a photodynamic reaction takes place.

Singlet oxygen should be involved in this reaction: in fact the rate of reaction in D_2O buffer, where singlet oxygen has a lifetime ten-fold increased [11], is faster than in H_2O buffer. Furthermore the rate of the reaction depends on the concentration of sensitizer. Finally it seems evident that singlet oxygen should be produced outside or on the surface of the micelles and it can migrate into the phospholipid bilayer because the energy transfer cannot take place from the sensitizer to the substrate without the singlet oxygen. Further experiments were carried out with diphenylfurane containing liposomes, one sample was prepared in buffer, the other in the porphyrin complex, and the results obtained are summarized in Fig. 4. Only in the presence of the complex the photodynamic reaction takes place, with an analogous behaviour to the systems previously described. With this kind of model systems such useful informations could be obtained in future similar experiments. Changing the composition and then the fluidity of the phospholipid bilayer (by introducing new components or by changing the nature of the hydrocarbon chains in the bilayer) the diffusion velocity of the singlet oxygen could be enhanced or reduced and the influence of the diffusion on the reactivity could be determined (paper in preparation).

An other interesting point we have begun to study is the determination of the peroxidation reactions due to irradiation in the presence of singlet oxygen, omitting the addition of the substrates while preparing hemoglobin porphyrin containing liposomes with a well defined hydrocarbon chain unsaturation. Preliminary results have been obtained that support the hypothesis of degradative oxidations of the lipid matrix and the consequent change in membrane structure and fluidity [12], consistent with the increased fragility of pathological red blood cells [13–16].

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