Light-induced Protoporphyrin Release from Erythrocytes in Erythropoietic Protoporphyria

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ABSTRACT The photohemolysis of normal erythrocytes incubated with protoporphyrin is reduced in the presence of albumin. When globin is added to normal erythrocytes loaded with protoporphyrin, protoporphyrin is bound to globin. During irradiation protoporphyrin moves from globin to the erythrocyte membrane and photohemolysis is initiated.

Erythrocytes in patients with erythropoietic protoporphyria contain large amounts of protoporphyrin bound to hemoglobin. Upon irradiation of these cells in the absence of albumin, 40% of protoporphyrin and 80% of hemoglobin is released after 240 kJ/m^2. The released protoporphyrin is hemoglobin bound. In contrast, when albumin is present only 8% of hemoglobin is released whereas protoporphyrin is released to 76%. The released protoporphyrin is albumin bound.

A hypothesis for the release of erythrocyte protoporphyrin in erythropoietic protoporphyria without simultaneous hemolysis is proposed: Upon irradiation protoporphyrin photodamages its binding sites on hemoglobin, moves through the plasma membrane, and is bound to albumin in plasma.

INTRODUCTION

Irradiation of erythrocytes from patients with erythropoietic protoporphyria results in photohemolysis of the cells (1, 2). This photohemolytic process has been characterized in a number of ways (3, 4). It is thought to be caused by oxidative cross-linking of membrane proteins (5) and (or) by photooxidation of cholesterol and membrane lipids (2, 6, 7). The toxic oxygen responsible for the photooxidative process is probably singlet oxygen (8). The importance of the photohemolysis in vivo is, however, unknown.

The most pronounced symptom in erythropoietic protoporphyria is cutaneous photosensitivity (9) and the skin of these patients contains increased amounts of protoporphyrin (10). Gschnait et al. (11) have proposed that protoporphyrin in the skin originates from the erythrocytes. It is known that the erythrocyte content of protoporphyrin diminishes as a function of the life time of the erythrocytes (12, 13). In the erythrocytes protoporphyrin is probably bound either to heme binding sites on globin (14, 15) or to hemoglobin not associated with the heme binding places (16). Patients with erythropoietic protoporphyria have no hemolytic anemia, thus protoporphyrin has to be released from the erythrocytes independent of hemoglobin.

The present paper proposes a mechanism on how protoporphyrin can escape from erythrocytes without simultaneous hemolysis.

METHODS

Erythrocytes were obtained from healthy persons or from patients with erythropoietic protoporphyria. Erythropoietic protoporphyria was diagnosed from a history of skin sensitivity to light, the findings of typical skin lesions on light exposed areas and erythrocyte protoporphyrin above 40 μM (normal values: <2.0 μM). The erythrocytes were washed three times with 0.15 M NaCl and diluted to a final concentration of 8×10^{10} cells/liter. Loading of normal cells with protoporphyrin was carried out by incubating the cells (after dilution) with 0.5 μM protoporphyrin for 10 min at 22°C. After this incubation 90–95% of the added protoporphyrin was taken up by the erythrocytes as estimated from the amount of protoporphyrin in the supernatant and pellet after centrifugation (800 *g*, 10 min). During the first few hours after protoporphyrin addition, protoporphyrin is located to the erythrocyte membrane as seen from the fluorescence emission peak of protoporphyrin at 636 nm. (Table II). Later it is bound to hemoglobin in the cells (17). The erythrocytes were not washed after the addition of protoporphyrin. Further additions were as indicated in the legends to the figures. The pH of the suspension was 7.3.

The diluted cells were taken up in a cuvette 4 × 4 cm, light path 3 mm and irradiated with a photochemotherapy unit PUVA (H. Haldman, D 722, Schwenningen, West Germany) containing 14 fluorescence tubes F8 T5/BL PUVA Sylvania) in a bank. About 70% of the emission energy of these lamps is between 340 and 380 nm. The light intensity

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was 66 W/m² as measured at sample level with a UDT model 80X optometer equipped with a radiometric filter (United Detector Technology, Inc., Santa Monica, CA). The temperature was 22°C. The irradiation dose was from 0 to 240 kJ/m² corresponding to an irradiation time of 0 to 60 min. After irradiation the cells were left in the dark for 30 min and, unless otherwise indicated, centrifuged (800 *g*, 10 min). The release of hemoglobin and protoporphyrin were determined as percentage of the content in the sample prior to centrifugation.

Protoporphyrin was determined by extracting the samples with ethyl acetate/acetic acid (3:1 vol/vol). The porphyrins were thereafter transferred to 3 M HCl and determined fluorometrically as described by Piomelli et al. (18) using the excitation and emission wavelengths at 408 nm and 608 nm respectively and protoporphyrin as standard. The concentration of globin was given as globin monomers.

Chemicals. Protoporphyrin was obtained from Porphyrin Products (Utah). Human albumin was obtained from Kabi (Stockholm, Sweden). Human globin was purchased from ICN Pharmaceuticals (Cleveland, OH). Other chemicals were of highest purity commercially available. Double quartz distilled water was used throughout.

RESULTS

The fluorescence spectrum of protoporphyrin differs depending on the structures protoporphyrin is bound to (18–20). Thus, protoporphyrin bound to human albumin or to lipoprotein membranes has an emission peak at 636 nm (excitation wavelength 397 nm), whereas protoporphyrin bound to free globin or to hemoglobin has the fluorescence emission peak at 627 nm (Fig. 1). The fluorescence intensity of protoporphyrin is greater in the presence of globin than in the presence of albumin. This ratio can not be altered significantly by increasing the albumin concentration or altering the excitation wavelength.

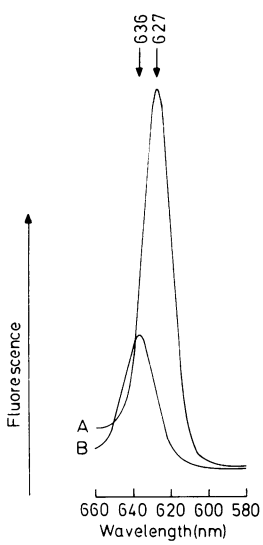

FIGURE 1 Fluorescence spectra of 0.5 μM protoporphyrin (suspended in 0.15 M NaCl, pH 7.3) in the presence of 1.0 μM globin (A) and 1.0 μM albumin (B). The excitation wavelength was 397 nm.

TABLE I
Fluorescence Emission Maxima of Protoporphyrin before and after Irradiation in the Presence of Globin and Albumin

	Emission maximum
	nm
A	627
B	629
C	636
D	627
E	627

Protoporphyrin (0.9 μM) in 0.15 M NaCl, pH 7.3 was incubated in the presence of globin (1.0 μM) (A) and irradiated 16 kJ/m² (B). In some experiments albumin (25 μM) (C) or albumin (25 μM) and thereafter globin (1.0 μM) (D) were added after irradiation. Nonirradiated protoporphyrin-globin with albumin added is given as (E). The excitation wavelength was 397 nm.

Irradiation of protoporphyrin-globin causes little change in the fluorescence emission spectrum of protoporphyrin (Table I), but the fluorescence intensity is greatly diminished (results not shown). After irradiation, addition of albumin (25 μM) changes the fluorescence emission top of protoporphyrin to 636 nm, whereas addition of albumin before irradiation causes no changes in the fluorescence spectrum. If globin (1 μM) is added to the irradiated protoporphyrin-globin supplemented with albumin, the fluorescence is increased and the emission maximum of protoporphyrin is again at 627 nm (Table I).

Erythrocytes incubated with protoporphyrin have the fluorescence emission peak at 636 nm (Table II) indicating that the majority of protoporphyrin is bound to erythrocyte membranes (at least within 1 h after the addition of protoporphyrin) (17). Upon photohemolysis of the cells (Fig. 2a), the fluorescence emission peak in the supernatant is at 627 nm, indicating that some of the added protoporphyrin is bound to hemoglobin released from the erythrocytes, i.e. after irradiation 240 kJ/m² ~30% of the total protoporphyrin is bound to hemoglobin in the supernatant. When albumin is added to the erythrocytes incubated with protoporphyrin, protoporphyrin is bound to albumin (Table II) and upon irradiation, the hemolysis is greatly decreased (Fig. 2b compared to Fig. 2a).

If globin is added to erythrocytes incubated with protoporphyrin, protoporphyrin will bind to globin (Fig. 3 and Table II). Upon irradiation, the amount of protoporphyrin bound to globin diminishes and photohemolysis is initiated (Fig. 3). The lower the globin

TABLE II

Fluorescence Emission Maxima of Protoporphyrin before and after Irradiation of Erythrocytes Loaded with Protoporphyrin or Erythrocytes from Patients with EPP

	Emission maximum	
	Supernatant	Pellet
	nm	
Protoporphyrin + erythrocytes	—	636
Protoporphyrin + erythrocytes irradiated 240 kJ/m^2	627	636
Protoporphyrin + erythrocytes + albumin	636	636
Protoporphyrin + erythrocytes + albumin irradiated 240 kJ/m^2	636	636
Protoporphyrin + erythrocytes + globin	627	—
Protoporphyrin + erythrocytes + globin irradiated 240 kJ/m^2	627	636
Erythrocytes from EPP patients	627	627
Erythrocytes from EPP patients irradiated 240 kJ/m^2	627	627
Erythrocytes from EPP patients + albumin	627	627
Erythrocytes from EPP patients + albumin irradiated 240 kJ/m^2	636	627

Erythrocytes incubated with protoporphyrin (0.5 μM) or erythrocytes from patients with EPP (erythrocyte protoporphyrin from 54 to 180 μM) were washed three times, diluted to 8 × 10^{10} cells/liter with 0.15 M NaCl and irradiated in the presence of albumin (15 μM) or globin (0.50 μM). The fluorescence spectra were recorded in the supernatants and pellets after centrifugation (800 *g*, 10 min). The excitation wavelength was 397 nm.

concentration, the quicker is the photohemolysis of the erythrocytes (Table III).

In patients with erythropoietic protoporphyria, erythrocytes contain large amounts of protoporphyrin (21). When irradiated, hemoglobin together with protoporphyrin escape from the cells (Fig. 4a). Protoporphyrin in the supernatant is hemoglobin bound as seen from the fluorescence emission maximum at 627 nm (Table II). When albumin is added, the release of protoporphyrin from the erythrocytes is increased and precedes the release of hemoglobin (Fig. 4b). The released protoporphyrin has a fluorescence maximum at 636 nm (Table II) indicating binding to albumin. After equilibration of the supernatant (from the sample irradiated 240 kJ/m^2) at 22°C for 24 h in dark, this fluorescence maximum has shifted to 627 nm.

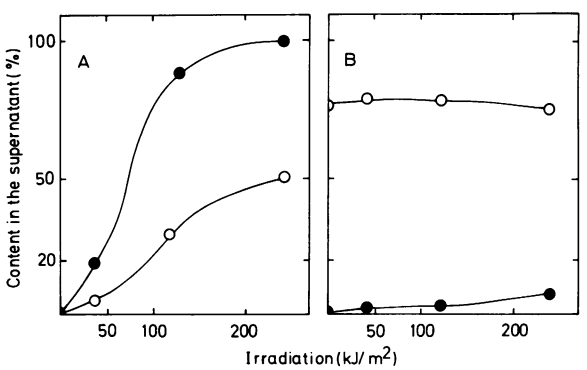

FIGURE 2 Effect of albumin on the photo-induced release of protoporphyrin (O) and hemoglobin (●) from normal erythrocytes incubated with 0.5 μM protoporphyrin. Normal erythrocytes were washed three times, diluted, and incubated with 0.5 μM protoporphyrin. After incubation for 10 min, 90–95% of the added protoporphyrin was taken up by the erythrocytes. Protoporphyrin was bound to the erythrocyte membrane (Table II and ref. 17). The suspension was irradiated in the absence (A) and in the presence (B) of 15 μM albumin. Irradiation temperature was 22°C. After irradiation, the cells were dark incubated for 30 min. The percentage of protoporphyrin (O) and hemoglobin (●) in the supernatant were recorded after centrifugation (800 *g*, 10 min).

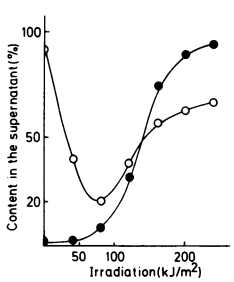

FIGURE 3 Effect of globin on protoporphyrin-induced photohemolysis. Normal erythrocytes were incubated and irradiated in the presence of 0.5 μM protoporphyrin and 0.50 μM globin. The percentage of protoporphyrin (O) and hemoglobin (●) in the supernatant were recorded after centrifugation. Experimental conditions were as in Fig. 2.

TABLE III
Effect of Different Amounts of Globin on Protoporphyrin-induced Photohemolysis

Globin	Irradiation doses necessary for 50% photohemolysis
μM	kJ/m^2
0	60
0.30	80
0.50	140
2.0	200
10.0	>240

Normal erythrocytes were incubated and irradiated in the presence of 0.5 μM protoporphyrin and different amounts of globin. The irradiation doses necessary to obtain 50% photohemolysis were recorded.

Dark incubation of erythrocytes (in the presence of albumin) from patients with erythropoietic protoporphyria (EPP)[1] for 24 h at 22°C causes <1% release of hemoglobin and protoporphyrin. The fluorescence emission peak of protoporphyrin in the supernatant after dark incubation is still at 627 nm.

Protoporphyrin is photooxidized during irradiation (22). At 240 kJ/m^2 the protoporphyrin concentration is about half the value of the nonirradiated sample. The nature of the photooxidation products of protoporphyrin has not been determined.

DISCUSSION

The photosensitivity in EPP is caused by protoporphyrin present in the skin (9, 10). The origin of the skin protoporphyrin is unknown although most authors believe that cells in the skin accumulate protoporphyrin released from the erythrocytes (11, 13). The mechanism of the release of protoporphyrin from erythrocytes, the transport and uptake by endothelial, dermal, or epidermal cells is largely unknown. The erythrocyte protoporphyrin content declines as a function of the life time of the erythrocyte (12, 13), Piomelli et al. (13) showed that ~10% of the total erythrocyte protoporphyrin escaped from the erythrocytes when they were incubated in the dark with compatible plasma for 18 h, pH 7.5, and temperature 37°C. There was no hemolysis. However, no protoporphyrin release was present when erythrocytes were incubated at pH 5.7. In our system, the release of protoporphyrin or hemoglobin from EPP erythrocytes is not increased after dark incubation for 24 h at 22°C.

Upon irradiation of protoporphyrin-globin, protoporphyrin probably photodamages the heme binding sites on globin and is bound either unspecific to globin or to other structures. The fluorescence is diminished. With the addition of albumin, protoporphyrin is bound to albumin (fluorescence emission peak at 636 nm) and upon addition of new globin most of the protoporphyrin moves to globin and the fluorescence is again at 627 nm (Table I). These findings are of importance in understanding the changes in the fluorescence emission spectrum of protoporphyrin released from erythrocytes in patients with EPP.

That protoporphyrin can photodamage globin was shown by Treffrey and Ainsworth (22): nearly all histidine and tryptophane residues and two-thirds of all methionine residues in globin were destroyed after irradiation of protoporphyrin-globin. They also suggest that the residues that lie in close proximity to the porphyrin ring are the first to be photodamaged (22). Light-induced dissociation of protoporphyrin from hemoglobin is supported by the studies of de Goeij et al. (14) who showed that the amount of protoporphyrin not associated with hemoglobin increased after blood samples from patients with erythropoietic protoporphyria had been exposed to light.

Albumin is supposed to be the most important protoporphyrin carrier in the plasma (23). With the concentrations used in the present study, which simulate those in plasma, albumin has a greater binding capacity for protoporphyrin than has the erythrocyte membrane (Fig. 2). The consequence of this albumin binding is that the protoporphyrin-induced photohemolysis is diminished (Fig. 2b compared to Fig. 2a). This is in agreement with the results from Joenje et al. (24) who found decreased photohemolysis of erythrocytes from patients with EPP in the presence of serum or plasma and with the results from Moan et al. (25) who

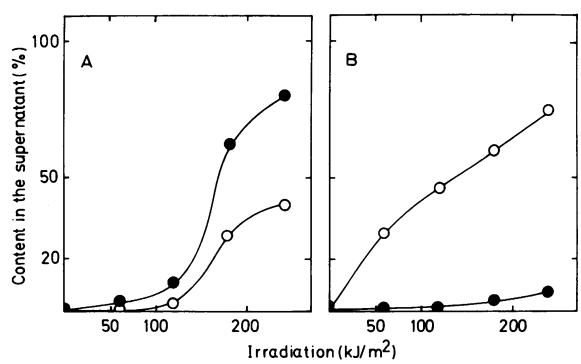

FIGURE 4 Effect of albumin on the photo-induced release of protoporphyrin (○) and hemoglobin (●) from erythrocytes from a patient with EPP. The erythrocytes were washed, diluted, and irradiated in the absence (A) and in the presence (B) of 15 μM albumin. The EPP concentration was 120 μM.

[1] *Abbreviation used in this paper*: EPP, erythropoietic protoporphyria.

found decreased photosensitizing efficacy of porphyrins in the presence of serum.

In erythropoietic protoporphyria Lamola et al. (16) propose that protoporphyrin in the erythrocytes is bound to intersubunit places on hemoglobin, whereas van Steveninck et al. (15) suggest that protoporphyrin is bound to heme binding sites on globin. In the presence of globin, however, protoporphyrin is bound on the heme binding sites (16, 22). In the present experiments (Fig. 3 and Table I) ~90% of exogenously added protoporphyrin is bound to globin. Upon irradiation protoporphyrin probably photodamages the heme binding sites on globin and moves to the erythrocyte membrane, whereupon photohemolysis is initiated (Fig. 3 and Tables II, III).

At equimolar concentrations of protoporphyrin, photohemolysis is much more severe in normal erythrocytes incubated with protoporphyrin than in erythrocytes from patients with erythropoietic protoporphyria (26). This can be explained by assuming that exogenously added protoporphyrin is localized to the membrane (at least after the short time incubation used here) (Table II) and that it is distributed equally between the erythrocytes. The porphyrin-induced photodamage is supposed to occur in the vicinity of the porphyrin molecule (24). This together with our results leads to the following hypothesis concerning the mechanism of protoporphyrin release from erythrocytes in EPP: Upon irradiation, protoporphyrin damages its binding sites on hemoglobin (Table I and Fig. 3), whereafter it preferentially binds to lipoprotein membranes, e.g. the plasma membrane. When albumin is present in the medium, protoporphyrin moves from the plasma membrane to albumin because the affinity of protoporphyrin to albumin is greater than its affinity to the plasma membrane (Fig. 2b). The photohemolysis is therefore less in the presence of albumin than in the absence of albumin (Fig. 2, 4). When erythrocytes from patients with EPP are irradiated, the release of protoporphyrin in the presence of albumin (Fig. 4b) can not be accounted for by assuming that only the most protoporphyrin-rich cells are photohemolyzed because the amount of protoporphyrin released compared to the hemolysis is too high (13), i.e. at 60 kJ/m^2 the release of protoporphyrin is 26.3% (increase from 0.5%), whereas the hemolysis is not significantly increased. Additionally, the fluorescence emission peak of the released protoporphyrin at 636 nm suggests that protoporphyrin is bound to albumin. After equilibration for 24 h, the released protoporphyrin redistributes and is bound to hemoglobin if hemoglobin is released in sufficient amounts. The redistribution of protoporphyrin to hemoglobin is concentration dependent and takes place if the albumin concentration is <40 times the hemoglobin concentration (27). The concentration of albumin here is 15 μM and of hemoglobin 3.4 μM (8% hemolysis after 240 kJ/m^2). Thus the immediate binding of protoporphyrin to albumin suggests that protoporphyrin is released independent of hemoglobin. In vivo, the hemolysis is negligible and protoporphyrin remains bound to albumin.

Whether the above mentioned mechanism can hold in vivo or not remains to be seen. The light intensity of near ultraviolet light (315–400 nm) reaching the dermis on a bright sunny day can be calculated to be ~20 W/m^2 (28). To obtain 20% release of protoporphyrin 40 kJ/m^2 is necessary in our in vitro system. This corresponds to ~30 min in bright sunshine. That the erythrocyte protoporphyrin content in patients with EPP is greater in winter than in summer (29) may support our hypothesis. It is, however, difficult to compare our in vitro system with irradiation of dermal capillaries in vivo. Nevertheless, the results may contribute to the understanding of the mechanism of the protoporphyrin release from erythrocytes in EPP.

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