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Protective Role of Pentoxifylline and Propentofylline Against the Increase in Osmotic Fragility of the Human Red Cell Induced by Protoporphyrin Photosensitization

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ABSTRACT

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Pentoxifylline and propentofylline are potent drugs used in impaired blood-flow pathologies. As an approach to understand their mode of action we studied their influence on protoporphyrin-photosensitized damage of the human red cell membrane. Both compounds are shown to exert a strong protection against the increase in osmotic fragility of the red cell which follows their irradiation at 390 nm in the presence of protoporphyrin. They do not, however, prevent cross-linking of the membrane skeleton proteins, which occurs slowly following irradiation. The results emphasize the ability of pentoxifylline and propentofylline to scavenge toxic photoactivated radicals, a property which could explain their mode of action in vivo. We also show that the two methyl xanthine derivatives considered in this study significantly protect red cells from hemolysis and spontaneous microvesiculization of their plasma membrane, which normally occurs when whole blood is stored for several weeks.

Key words: membrane photooxidation, hemolysis, protoporphyrin

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Abbreviations used: PBS, phosphate buffer saline (5 mM sodium phosphate, 145 mM NaCl (pH 7.5)); PTF, pentoxifylline (3,7-dimethyl-1-(5-oxohexyl)-xanthine; PPF, propentofylline (3-methyl-1-(5-oxohexyl)-7-propyl-xanthine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

INTRODUCTION

Pentoxifylline (PTF) is a pharmacologically active drug used for treatment of blood-flow disturbances. A more recently synthesized related molecule, propentofylline (PPF), may represent in this area a still more efficient compound. Presently, the mechanisms whereby they exert their action are still not completely understood. Several targets are certainly involved, such as endothelial cells, platelets, erythrocytes, and leucocytes. The erythrocyte membrane is presumably involved since, in patients with characterized blood flow pathologies, PTF and PPF improve the deformability and filtrability of the red cells [Dormandy et al., 1981; Isogai et al., 1981; Seiffge and Nagata, 1985; Sowemimo-Coker and Turner, 1985].

In a recent study involving a broad set of different experimental approaches, we were not able to detect any effect of PTF and PPF on some of the structural and functional properties of the normal human erythrocyte membrane [Chetrite et al., 1988]. We have suggested that their therapeutic action could rely on protection against pathological, mainly oxidative, membrane stresses, rather than on their ability to restore the properties of the damaged red cell membrane.

In the light of numerous investigations of protoporphyrin-photosensitized effects on erythrocytes [Girotti, 1976; Lamola and Doleiden, 1980; Dadosh and Shaklai, 1988], we chose this experimental model to generate well-characterized oxidative stresses. The occurrence of these stresses in vivo, in patients with congenital erythropoietic porphyria [Harber et al., 1984; Taddeini and Watson, 1968], futher increases the interest of this model. This report represents an attempt to examine whether, in the protoporphyrin-induced oxidative attack of erythrocytes, leading to an increase in osmotic fragility of the cells and in cross-linking of their membrane skeleton proteins [Girotti, 1976; Lamola and Doleiden, 1980], a protective role of PTF and PPF can be demonstrated.

MATERIALS AND METHODS

PTF and PPF were obtained through the courtesy of Hoechst-France Laboratories. Protoporphyrin disodium salt and caffeine were from Sigma. Freshly collected blood in citrate-dextrose medium was obtained from the Centre National de la Transfusion Sanguine (Paris). The erythrocytes were washed three times in phosphate-buffered saline (PBS) by centrifugation at 1,000*g* for 10 min, and buffy coat was removed at each step. One volume of the washed packed erythrocytes was diluted in nine volumes of PBS containing 5 μM protophorphyrin, in the absence or in the presence of 1 mM PTF and PPF. The red cell suspension was continuously and gently stirred in a flat vessel under one atmosphere of air and at 20°C. It was uniformly irradiated for 30 min with a 400 W Xenon lamp, through a converging lens and a blue broad band filter (λ_{max} = 390 nm with a pathband of 100 nm measured at half-peak transmission). An infrared filter was used to prevent heating of the sample. The setting of the irradiating system was precisely controlled in order to ensure reproducible results.

After irradiation, aliquots of the red cell suspension were diluted tenfold in a range of concentrations of PBS in water and centrifuged 10 min at 4,500*g*. The absorbances A_{540} and A_{578} of the supernatants were measured at 540 nm and 578 nm. The corresponding values $A_{540,T}$ and $A_{578,T}$ found for complete hemolysis of the red cells in water were used to determine the percentage of hemolysis according to the relations $(A_{540}/A_{540,T}) \times 100$ and $(A_{578}/A_{578,T}) \times 100$. The time of irradiation was such that no spontaneous hemolysis of the red cells in isotonic medium was observed. Cross-linking of the red cell membrane proteins was analyzed by SDS-PAGE [Laemmli, 1970] by using a 5–15% polyacrylamide gradient in the resolving gel and a 5% stacking gel. The red cell membranes were prepared by lysis of one volume of the irradiated cells in ten volumes of 5 mM phosphate buffer, pH 7.7, containing phenylmethylsulfonyl fluoride 1 mM, at 4°C, followed by centrifugation at 20,000*g* for 20

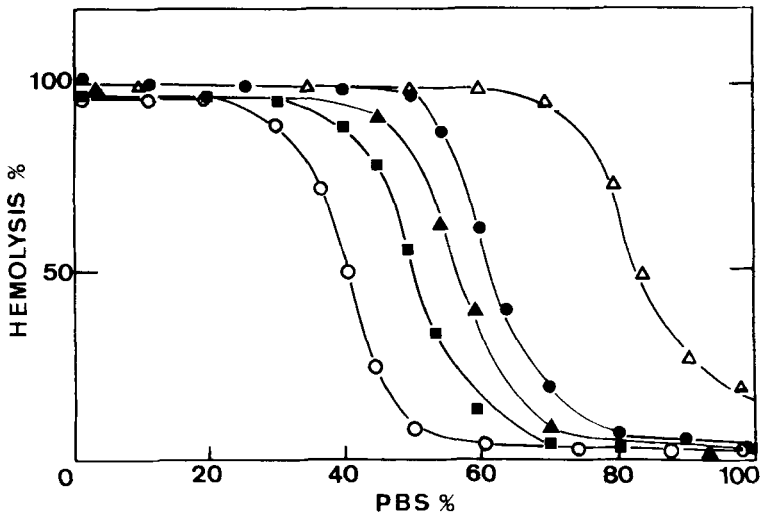

Fig. 1. Influence of the xanthine derivatives (PTF, PPF, and caffeine) on hemolysis of a suspension of human red cells photoirradiated at 390 nm in the presence of protoporphyrin. The experiments were performed immediately after irradiation of the cells. The PBS concentration in water was varied between 0 and 100%. The percentage of hemolysis was determined as described in Materials and Methods. Protoporphyrin 5 μM was present in all the samples. Hemolysis of the red cells without irradiation ○; after irradiation △ after irradiation in the presence of PTF ●, PPF ■, caffeine ▲ (1 mM). In the absence of protoporphyrin hemolysis of the red cells was independent of irradiation and identical to the experiment shown with open circles.

min. The pellets were washed several times in the same buffer until free of hemoglobin. The protein content of the membranes was evaluated according to the method of Lowry et al. [1951]. The SDS gels were stained with Coomassie blue [Laemmli, 1970].

Long-term storage of the whole blood of a single donor was made in the dark and at 4°C in the absence and in the presence of PTF and PPF (1 mM). Spontaneous micro-vesiculization of the red cells membrane, which occurs progressively in the process of storage, was estimated by recovering from the plasma two populations of vesicles by differential centrifugation: those sedimenting between 7,000*g* and 20,000*g* and those sedimenting between 20,000*g* and 100,000*g* [Rumsby et al., 1977]. Subsequently, the pellets were washed once in PBS at 4°C, recovered by centrifugation, and resuspended in a given volume of PBS. Transmission of the vesicle suspensions was measured spectrophotometrically at 700 nm in a 0.5 cm pathlength optical cuvette. The ratio of the values found for 7 weeks-stored blood in the absence of any additive or in the presence of PTF or PPF was indicative of the relative amounts of vesicles generated during storage.

RESULTS

Figure 1 shows the relationship between PBS concentration in water and lysis of a suspension of erythrocytes immediately after irradiation in the absence and in the presence of protoporphyrin, PTF, and PPF. Irradiation in the presence of protoporphyrin alone dramatically decreases the hypotonic resistance of the red cells. PTF exerts a strong protection which was even stronger with PPF. Caffeine, a related molecule, was also tried and gave an intermediate effect. The results were dose-dependent: a lower protection of erythrocytes was

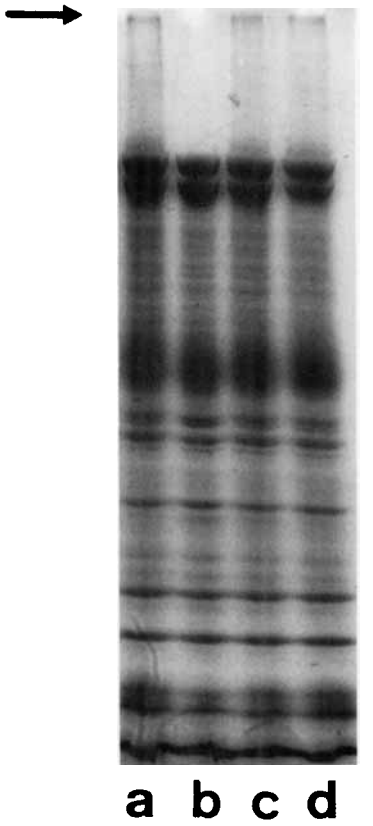

Fig. 2. SDS-PAGE of human erythrocyte membranes prepared from human red cells after irradiation at 390 nm in the presence (**a**) and in the absence (**b**) of protoporphyrin and in the presence of protoporphyrin and PTF (**c**) or PPF (**d**). Aggregates of cross-linked proteins are shown by an arrow at the top of the gel. They are totally absent in lane b.

noticed after a tenfold dilution (100 μM) of the drugs (not shown). Aliquots of the irradiated cells were processed for SDS-PAGE electrophoresis of their membrane proteins (Fig. 2). As previously described [Lamola and Doleiden, 1980; Dadosh and Shaklai, 1988; Deziel and Girotti, 1980], the appearance of a high molecular weight component at the top of the gel reflects a light-dependent cross-linking of their membrane and skeleton proteins. PTF and PPF were unable to prevent occurrence of cross-linking (Fig. 2).

Table 1 also shows a beneficial effect on autohemolysis and micro-vesiculization of the red cells after 7 weeks storage of the blood. Hemolysis was decreased twofold; the fraction of micro-vesicles which could be pelleted at 20,000*g* dropped by about 25% and the smaller-size vesicles, which correspond to the 100,000*g* sedimenting population, were no longer found.

DISCUSSION

Blue irradiation of erythrocytes in the presence of protoporphyrin [Girotti, 1976; Lamola and Doleiden, 1980; Dadosh and Shaklai, 1988] or several other heme derivatives [Sorata et al., 1988; Girotti, 1978; Deziel and Girotti, 1980] lowers their osmotic resistance. A second, slow, process occurs within the irradiated cells which results in complete hemolysis after

TABLE 1. Protective Role of PTF and PPF Against Spontaneous Hemolysis and Micro-Vesiculization of Several-Weeks-Stored Whole Blood*

	Spontaneous hemolysis (percent)	Microvesicles	
		20,000*g*	100,000*g*
Freshly collected blood	0	0	0
Seven weeks-stored blood	5.7 ± 0.5	100	traces
Seven weeks-stored blood with PTF (1 mM)	2.5 ± 0.3	76 ± 7	0
Seven weeks-stored blood with PPF (1 mM)	2.4 ± 0.4	70 ± 8	0

*The columns below microvesicles refer to the two populations of vesicles sedimenting at different accelerations. The amounts of microvesicles generated in the presence of PTF and PPF are expressed as percent of the maximal amounts obtained for the seven weeks-stored blood in the absence of any additive (row 2). The values shown in the table represent the average of three independent experiments. They are given with standard deviation values.

several hours in the dark. The development of membrane damage in erythrocytes when the source of the effect (blue irradiation) has disappeared was also shown with the radical forming oxidant t-butylhydroperoxide [Deuticke et al., 1987]. We have considered in this study only the first of these two processes by choosing appropriate light intensity and time of irradiation and by measuring the osmotic resistance of the cells and cross-linking of their membrane proteins immediately after irradiation.

Several groups using spin trapping, electron paramagnetic resonance, and laser flash kinetic spectroscopy techniques have demonstrated that in the presence of light and oxygen, aqueous solutions of protoporphyrin and several other heme derivatives are the source of singlet oxygen, superoxide, and hydroxyl radicals [Buettner and Oberley, 1980; Faraggi et al., 1984; Firey and Rodgers, 1988]. These toxic species severely damage the red cell membrane through lipid peroxidation [Rumsby et al., 1977; Sorata et al., 1988], cross-linking of membrane skeleton proteins [Lamola and Doleiden, 1980; Dadosh and Shaklai, 1988; Girotti, 1978], changes in the conformation of transmembrane band 3 protein, and inhibition of its anion transport activity [Dubbelman et al., 1981]. Membrane protein cross-linking and red cell oxidative photohemolysis are not coupled processes [Lamola and Doleiden, 1980].

This report demonstrates that PTF and PPF protect human red cells against lysis but not against membrane protein cross-linking (Figs. 1, 2), as was also shown for the antioxidant butylated hydroxytoluene [Deziel and Girotti, 1980]. This suggests that lipid peroxidation is a crucial factor involved in the osmotic resistance of the red cell membrane and that the therapeutic action of PTF and PPF may rely in their ability to scavenge lipid radicals inside the red cell membrane. This implies that PTF and PPF should be continuously present inside the lipid bilayer. Recent studies on the permeability of the erythrocyte membrane to PTF and PPF shows this is indeed the case [Chetrite et al., 1988]. These molecules do not penetrate in the red cells through the water-carrying pores of their membrane. Instead, they freely diffuse through the whole lipid bilayer. The kinetics of the inward and outward flux are rapid, implying a fast turnover for the presence of PTF and PPF in the membrane. This represents a prerequisite condition for the two drugs to act, at therapeutic doses, as radical scavengers of toxic radical species generated in the densely packed lipid domains of the erythrocyte membrane.

The protoporphyrin-induced sensitization of the red cells to hemolysis is less pronounced in the presence of PPF than PTF (Fig. 1). This difference cannot be attributed to the hydrophobic character of the 7-propyl substituent on the xanthine ring of PPF since caffeine with 1- and 7-methyl groups gives an intermediate effect. It is more probably due to differences in the intrinsic electrophilic character of PTF and PPF, but this point deserves further studies.

Our results suggest the use of PTF and PPF in pathological cases associated with an increase in oxidative stresses. This is particularly true for patients with altered blood filtrability. In sickle cell disease and in unstable hemoglobin-linked hemolytic anemia, production of toxic radicals is well documented [Van den Berg et al., 1988; Platt and Falcone, 1988]. PTF and PPF may also be particularly relevant in phototherapy of neonatal hyperbilirubinemia and in photodynamic therapy [Kopelman et al., 1972; Manyak et al., 1988; Athar et al., 1988]. The amounts of PTF and PPF which were used in this study represent five to ten times more than the normal levels achievable in vivo [*Dictionnaire Vidal,* OVP Edit. Paris, 1986, p. 1544]. However, one has to consider that in pathological cases of blood flow disturbances, the amount of toxic radicals which are formed is certainly lower than in the experimental conditions of this work, i.e., normal red cells subjected in vitro, in a serum-free medium, to drastic photochemical stresses.

Since lipid peroxidation of the red cell membrane occurs in the process of cellular aging [Kay et al., 1986; Jain, 1988], we have been examining the possibility that PTF and PPF protect the red cell during long-term storage of blood. Table 1 indicates significant protection by the two drugs against spontaneous hemolysis and membrane fragmentation in microvesicles. This implies that PTF and PPF may represent beneficial additives for storage of the blood. A systematic study in this direction is worth considering.

In conclusion, we suggest a mechanism for the therapeutic action of PTF and PPF. These compounds most probably act as toxic radical scavengers, as do tocopherol, quercetin, butylated hydroxytoluene, and uric acid, which interrupt the chain of peroxidative reactions inside biological membranes [Miki et al., 1987; Sorata et al., 1988; Deziel and Girotti, 1980; Niki et al., 1988]. Blood plasma is poor in antioxidant compounds and the red cells are known to scavenge extracellular hydrogen peroxide and superoxide radicals [Winterbourn and Stern, 1987]. In some pathological cases, the amounts and reactivity of these compounds may be too large for the red cells to efficiently buffer their toxicity. In these conditions, PTF and PPF can represent very potent active drugs. Recent studies have also indicated their influence on white cell functions: in some of the cases studied, the implication of their antioxidant character is emphasized [Flechter et al., 1987; Freyburger et al., 1987].

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REFERENCES

Athar, M., Mukhtar, H., Elmets, C.A., Tarif Zaim, M., Lloyd, J.R., and Bickers, D.R.: In situ evidence for the involvement of superoxide anions in cutaneous porphyrin photosensitization. Biochem. Biophys. Res. Commun. **151:**1054–1059, 1988.

Buettner, G.R. and Oberley, L.W.: The apparent production of superoxide and hydroxyl radicals by hematoporphyrin and light as seen by spin-trapping. FEBS Lett. **121:**161–164, 1980.

Chetrite, G., Lebbar, I., and Cassoly, R.: Etude de la perméabilité de la membrane érythrocytaire à la pentoxifylline et la propentofylline. Recherche de leurs effets sur quelques propriétés structurales et fonctionnelles de la membrane. Pathol. Biol. (Paris) **36:**1066–1072, 1988.

Dadosh, N. and Shaklai, N.: Impairment of red cell membrane cytoskeleton by protoporphyrin-IX: light and dark effects. Photochem. Photobiol. **47:**689–697, 1988.

Deuticke, B., Heller, K.B., and Haest, C.W.M.: Progressive oxidative membrane damage in erythrocytes after pulse treatment with t-butylhydroperoxide. Biochim. Biophys. Acta **899:**113–124, 1987.

Deziel, M.R. and Girotti, A.W.: Bilirubin-photosensitized lysis of resealed erythrocyte membranes. Photochem. Photobiol. **31:**593–596, 1980.

Dormandy, J., Ernst, E., and Flute, P.: Increase in red cell filtrability after incubation with oxpentifylline. Curr. Med. Res. Opin. **7:**520–522, 1981.

Dubbelman, T.M.A.R., De Goeij, A.F.P.M., Christianse, K., and Van Steveninck, J.: Protoporphyrin-induced photodynamic effects on band 3 protein of human erythrocyte membranes. Biochim. Biophys. Acta **649:**310–316, 1981.

Faraggi, M., Carmichael, A., and Riesz, P.: OH radical formation by photolysis of aqueous porphyrin solutions. A spin trapping and e.s.r. study. Int. J. Radiat. Biol. **46:**703–713, 1984.

Firey, P.A. and Rodgers, M.A.J.: Photochemical properties of erythrocyte ghosts containing porphyrin. Photochem. Photobiol. **47:**615–619, 1988.

Fletcher, J., Slater, K., Wiseman, M.S., and Shale, D.J.: The effect of pentoxifylline on neutrophil function in vitro and ex vivo in human volunteers. Symposium on Pentoxifylline and Leukocyte Function. Key Biscayne, Florida: Abstracts, 1987, p. 13.

Freyburger, G., Hammerschmidt, D., Coppo, P., and Boisseau, M.R.: Inhibition of granulocyte responsiveness by pentoxifylline: hemorheological effects. Symposium on Pentoxifylline and Leukocyte Function. Key Biscayne, Florida: Abstracts, 1987, p. 10.

Girotti, A.W.: Photodynamic action of protoporphyrin IX on human erythrocyte cross-linking of membrane proteins. Biochem. Biophys. Res. Commun. **72:**1367–1374, 1976.

Girotti, A.W.: Bilirubin photosensitized cross-linking of polypeptides in the isolated membrane of the human erythrocyte. J. Biol. Chem. **253:**7186–7193, 1978.

Harber, L.C., Fleischer, A.S., and Baer, R.L.: Erythropoietic protoporphyria and photohemolysis. J.A.M.A. **189:**191–194, 1964.

Isogai, Y., Mochizuki, K., and Ashikaga, M.: A new method of measuring red cell deformability and the effects of pentoxifylline. Curr. Med. Res. Opin. **7:**352–358, 1981.

Jain, S.K.: Evidence for membrane lipid peroxidation during the in vivo aging of human erythrocytes. Biochim. Biophys. Acta **937:**205–210, 1988.

Kay, M.M.B., Bosman, G.J.C.G., Shapiro, S.S., Bendich, A., and Bassel, P.S.: Oxidation as a possible mechanism of cellular aging: Vitamin E deficiency causes premature aging and IgG binding to erythrocytes. Proc. Natl. Acad. Sci. U.S.A. **83:**2463–2467, 1986.

Kopelman, A.E., Brown, R.S., and Odell, G.B.: The "bronze" baby syndrome: a complication of phototherapy. J. Pediatr. **81:**466–471, 1972.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685, 1970.

Lamola, A.A. and Doleiden, F.H.: Cross-linking of membrane proteins and protoporphyrin-sensitized photohemolysis. Photochem. Photobiol. **31:**597–601, 1980.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J.: Protein measurement with the folin phenol reagent. J. Biol. Chem. **193:**265–275, 1951.

Manyak, M.J., Russo, A., Smith, P.D., and Glatstein, A.: Photodynamic therapy. J. Clin. Ocnol. **6:**380–391, 1988.

Miki, M., Tamai, H., Mino, M., Yamamoto, Y., and Niki, E.: Free radical chain oxidation of rat red blood cells by molecular oxygen and its inhibition by α-tocopherol. Arch. Biochem. Biophys. **258:**373–380, 1987.

Niki, E., Komuro, E., Takahashi, M., Urano, S., Ito, E., and Terao, K.: Oxidative hemolysis of erythrocytes and its inhibition by free radical scavengers. J. Biol. Chem. **263:**19809–19814, 1988.

Platt, O.S. and Falcone, J.F.: Membrane protein lesions in erythrocytes with heinz bodies. J. Clin. Invest. **82:**1051–1058, 1988.

Rumsby, M.G., Trotter, J., Allan, D., and Mitchell, R.H.: Recovery of membrane micro-vesicles from human erythrocytes stored for transfusion: A mechanism for the erythrocyte discocyte-to-spherocyte shape transformation. Biochem. Soc. Trans. **5:**126–128, 1977.

Seiffge, D. and Nagata, L.: Effects of propentofylline on the micromechanical properties of red blood cells. Drug Dev. Res. **5:**147–155, 1985.

Sorata, Y., Takahama, U., and Kimura, M.: Cooperation of quercetin with ascorbate in the protection of photosensitized lysis of human erythrocytes in the presence of hematoporphyrin. Photochem. Photobiol. **48:**195–199, 1988.

Sowemimo-Coker, S. and Turner, P.: The effect of pentoxifylline on filterability of normal red blood cells and their adhesiveness to cultured endothelial cells. Eur. J. Clin. Pharmacol. **29:**55–59, 1985.

Taddeini, L. and Watson, C.J.: The clinical porphyrias. Semin. Hematol. **5:**335–369, 1968.

Van den Berg, J.J.M., Kuypers, F.A., Qju, J.H., Chiu, D., Lubin, B., Roelofsen, B., and Op den Kamp, J.A.F.: The use of cis-parinaric acid to determine lipid peroxidation in human erythrocyte membranes. Comparison of normal and sickle erythrocyte membranes. Biochim. Biophys. Acta **944:**29–39, 1988.

Winterbourn, C.C. and Stern, A.: Human red cells scavenge extracellular hydrogen peroxide and inhibit formation of hypochlorous acid and hydroxyl radical. J. Clin. Invest. **80:**1486–1491, 1987.