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PROTOPORPHYRIN-INDUCED PHOTODYNAMIC EFFECTS ON TRANSPORT PROCESSES ACROSS THE MEMBRANE OF HUMAN ERYTHROCYTES

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Summary

Previous studies have shown that illumination of erythrocytes with visible light in the presence of protoporphyrin results in cross-linking of membrane proteins and deterioration of several membrane functions, e.g. active transport of K^+ and Na^+.

In the present study it is shown that carrier-mediated transport of glucose, L-leucine, sulphate and glycerol is also inhibited by the photodynamic process, whereas non-specific permeability of glycerol and thiourea is increased.

It is shown that these effects are not caused by lipid peroxidation, but by photooxidation of membrane proteins. The inhibition of carrier-mediated transport is caused either by photodynamic oxidation of susceptible essential amino acid residues of the carrier molecules, or by an aspecific perturbation of the membrane structure, leading to inhibition of carrier functions.

Introduction

Illumination of human erythrocytes with visible light in the presence of protoporphyrin as sensitizer, leads to extensive membrane damage, caused by direct photooxidation of membrane proteins [1—7]. This can be observed both with red blood cells of erythropoietic protoporphyria patients, which have an intracellular accumulation of protoporphyrin and with normal red blood cells, after addition of protoporphyrin to the incubation medium [8,9]. Probably singlet oxygen is involved in this photodynamic process [10,11]. The photodynamic process is characterized among other things by aggregation of membrane particles as seen by freeze-etch electron microscopy [3], inhibition of

membrane-bound enzymes [7,12], extensive cross-linking of membrane proteins [3,4,6,7] and, ultimately, colloid osmotic lysis [1,8].

In the prelytic phase active transport of Na^+ and K^+ is abolished, due to elimination of the ($Na^+ + K^+$)-dependent ATPase activity [9], whereas passive cation transport is strongly increased [8]. In the prelytic phase this leads initially to an approx. 1 : 1 exchange of Na^+ and K^+, without changes in cellular volume. When about 80% K^+ has leaked out of the cells, Na^+ influx begins to exceed K^+ efflux and, ultimately, osmotic lysis takes place [8].

In further experiments the photodynamic effects on carrier-mediated and aspecific solute transport during the prelytic phase were studied. The results are described in the present communication.

Methods

Heparinized human blood was centrifuged shortly after collection. The red blood cells were washed three times and resuspended in buffered isotonic NaCl solution (154 mM NaCl, 9.6 mM Na_2HPO_4 and 1.5 mM NaH_2PO_4). The red blood cells, suspended in 9 volumes of this medium, were illuminated in the presence of 0.2 mM protoporphyrin. Illumination was carried out under magnetic stirring of the suspension with a 125 W super high-pressure mercury lamp (Philips type HPL) placed at a distance of 10 cm from a lens. A parallel beam of light was reflected through the bottom of the incubation vessel by a mirror. The lamp has a strong emission band at 390—410 nm, the effective wavelength for protoporphyrin excitation.

The transport velocities of L-leucine, sulphate and D-glucose were assayed by measuring efflux of the radioactive substrate from preloaded cells, as described for amino acids by Winter and Christensen [13], for sulphate by Knauf and Rothstein [14] and for glucose by Van Steveninck et al. [15]. In these experiments the cells were loaded with labeled substrate before addition of protoporphyrin and illumination. Glycerol and thiourea permeability was measured by the hemolysis technique as described by Naccache and Sha'afi [16]. Aspecific glycerol transport was measured in the presence of 10^{-4} M Cu^{2+}, completely inhibiting the facilitated diffusion of glycerol [17].

K^+ in the medium was determined with a flame photometer. The effects of H_2O_2 on red blood cell permeability were studied after prior incubation of the cells with this agent as described by Stocks and Dormandy [18]. Malonaldehyde, the major secondary product of lipid peroxidation, was measured according to Ottolenghi [19].

Hemoglobin-free ghosts were prepared by the gradual osmotic lysis method of Weed et al. [20]. Polyacrylamide gel electrophoresis of membrane proteins was performed as described by Fairbanks et al. [21]. Densitometric scans of stained gels were recorded on a Zeiss PMQ II spectrophotometer with scanning device.

Results

The influence of irradiation of red blood cells with visible light in the presence of protoporphyrin on transmembrane transport of glucose is shown in

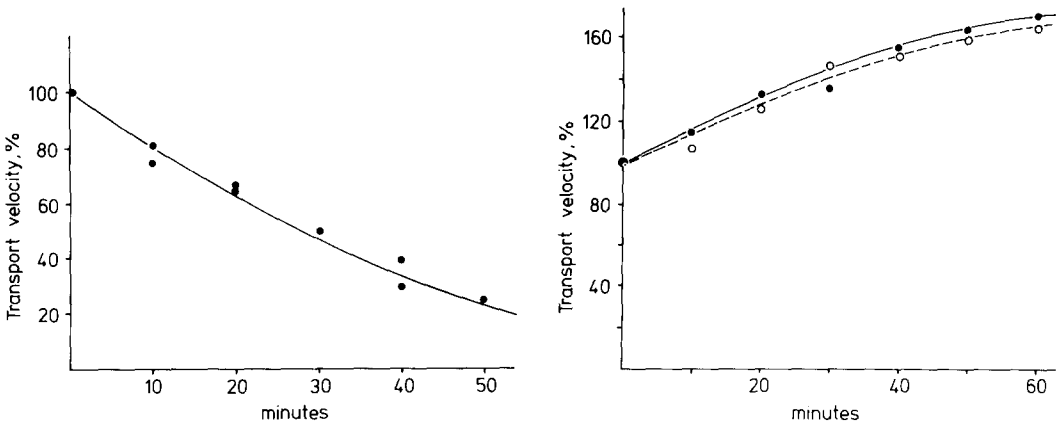

Fig. 1. The influence of illumination of red blood cells with visible light in the presence of protoporphyrin on glucose transport, under standard experimental conditions. The transport velocity is expressed in % of the initial rate.

Fig. 2. Photodynamic effects on the non-specific permeability of glycerol (○- - - - -○) and thiourea (●———●). The non-specific permeability of glycerol was measured in the presence of 10^{-4} M Cu^{2+}, to block carrier-mediated glycerol transport.

Fig. 1. Apparently glucose transport is progressively inhibited by the photodynamic process, with no lagtime. Carrier-mediated transport of leucine, sulphate and glycerol is affected in a similar way, as shown in Table I. After about 50 min the inhibition of glycerol transport is reversed.

The non-specific permeability of thiourea and glycerol on the other hand increased under these experimental conditions (Fig. 2). After an illumination period of about 50 min the velocity of non-specific glycerol influx (measured in the presence of 10^{-4} M Cu^{2+}) apparently exceeded the velocity of carrier-mediated glycerol influx. This explains the observed reversal of inhibition of glycerol transport in the absence of Cu^{2+} (Figs. 2 and 3).

The effect of the photodynamic process on the membrane proteins is shown in Fig. 4. In the prelytic phase an extensive oxidative cross-linking of mem-

TABLE I

THE INFLUENCE OF THE PHOTODYNAMIC PROCESS ON TRANSPORT OF GLUCOSE, LEUCINE, SULPHATE AND GLYCEROL IN THE PRELYTIC PHASE

Transport velocities are expressed in % of initial rates.

Illumination time (min)	Cellular K^+ (% of control)	Transport velocity (%)			
		Glucose	Leucine	Sulphate	Glycerol
0	100	100	100	100	100
10	97.6	80	88	78	81
20	94.3	63	60	58	65
30	90.1	47	44	50	45
40	80.1	31	34	39	25
50	73.3	20	17	22	10
60	61.7	14	10	15	21

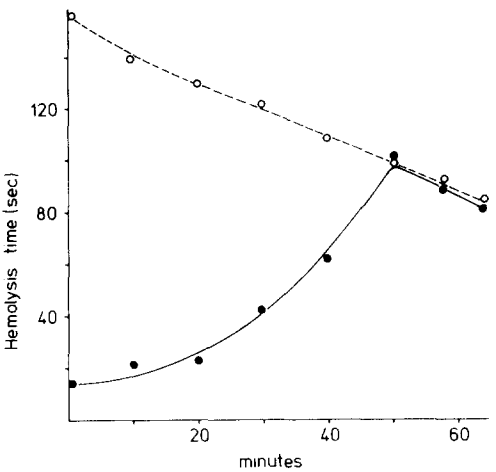

Fig. 3. Photodynamic effects on the hemolysis time of red blood cells in isotonic glycerol, in the absence (●——●) and in the presence (○- - - - -○) of 10^{-4} M Cu^{2+}.

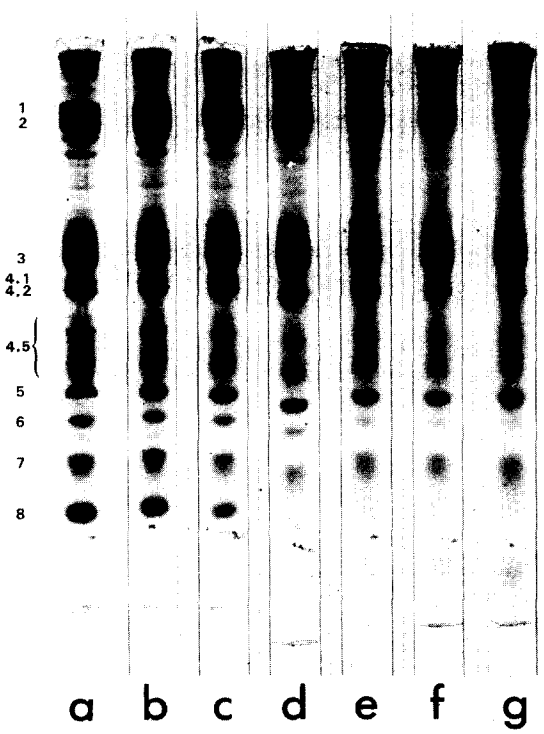

Fig. 4. Photodynamic cross-linking of membrane proteins during the prelytic phase. After the indicated illumination periods ghosts were prepared, followed by SDS-polyacrylamide gel electrophoresis of the solubilized membrane proteins. Illumination periods: (a) 0 min; (b) 10 min; (c) 20 min; (d) 30 min; (e) 50 min; (f) 60 min; (g) 70 min.

TABLE II

PHOTODYNAMIC CROSS-LINKING OF SOME MEMBRANE PROTEINS AND THE PHOTODYNAMIC INFLUENCE ON SULPHATE TRANSPORT

Both the amount of residual membrane proteins (as measured from densitometric scans of the gels) and the transport velocities are given in % of the initial values.

Illumination time (min)	Sulphate transport velocity (%)	Remaining protein (%)		
		Spectrin	Band 3	Band 6
0	100	100	100	100
10	82.1	87.2	93.2	80.3
20	60.3	72.2	91.7	60.4
35	48.2	45.3	87.5	22.4
50	22.1	23.4	78.4	12.8
60	15.0	15.9	76.2	8.4

TABLE III

THE INFLUENCE OF H_2O_2-INDUCED LIPID PEROXIDATION ON CARRIER-MEDIATED AND ON NON-SPECIFIC TRANSPORT OF GLYCEROL, AS COMPARED TO THE PHOTODYNAMIC INFLUENCE ON THESE PROCESSES

H_2O_2 (%)	Incubation time (min)	Malonaldehyde (μM)	Glycerol transport (%)	
			Carrier-mediated	Non-specific
0.06	15	4.3	100	100
0.06	30	7.8	99	97
0.06	45	10.4	99	92
0.20	15	23.6	97	96
0.20	30	32.8	80	103
0.20	45	50.7	76	120
photodynamic	50	0.2	10	158

brane proteins occurs, resulting in increasing amounts of high-molecular weight protein complexes on top of the gel. A concomitant decrease of protein bands 1, 2, 4.1 and 6 is observed, with a much slower decrease of band 3 and the 4.5 region. This was confirmed by quantitative scanning of the gels (Table II).

During illumination of the cells in the presence of protoporphyrin a very slow and limited peroxidation of unsaturated fatty acid side chains in the membrane takes place, leading to the formation of trace amounts of malonaldehyde. As shown previously, this peroxidation is not involved in the process of cross-linking of the membrane proteins [4]. It could not be concluded a priori, however, that the observed effects on transmembrane transport are also independent of this slow lipid peroxidation. Therefore the effects of H_2O_2, leading to a much more pronounced lipid peroxidation, on transmembrane transport of glycerol were measured. The results are shown in Table III.

Discussion

In previous papers it was shown that the photodynamic effect of protoporphyrin on the red cell membrane results in the deterioration of several mem-

brane functions, e.g. active transport of cations [9]. Further, a very slow and limited peroxidation of unsaturated fatty acid side chains in the membrane takes place. It could be demonstrated that this lipid peroxidation is not involved in the most striking effect of the photodynamic process: the oxidative cross-linking of membrane proteins [4]. It was shown that this cross-linking is probably caused by the secondary reaction between a photooxidation product of histidine residues and free NH_2 groups in the membrane proteins, yielding a covalent bond [22]. Besides histidine, methionine, cysteine, tyrosine and tryptophan are sensitive to photooxidative modification, but photooxidation of these residues does not lead to photodynamic cross-linking of proteins.

As shown in this paper facilitated diffusion of several substrates is inhibited by the photodynamic process, whereas non-specific permeation is increased. The experiments with H_2O_2 indicate that these photodynamic effects are not mediated by lipid peroxidation. As shown in Table III a H_2O_2-induced lipid peroxidation yielding a concentration of malonaldehyde 100 times higher than that found during illumination in the presence of protoporphyrin, has no effect on the studied transport processes. Therefore it seems likely that these photodynamic effects on transport are also caused by a direct photooxidation of membrane proteins.

It is highly probable that band 3 is involved in anion transport [23,24]. The percentage of sulphate transport inhibition exceeds the percentage of cross-linked band 3 molecules (Table II), suggesting that band 3 cross-linking is not primarily responsible for the observed transport inhibition. Two other possibilities should be considered. First, the transport inhibition can be caused by photooxidation of essential amino acid residues in band 3 protein, leading to impaired function but not to cross-linking. The second possibility would be an inhibition of transport, caused by a general deterioration of the membrane structure, as a consequence of the photodynamic process. It is as yet impossible to discriminate between these possibilities. The close parallel between inhibition of glucose, sulphate, glycerol and leucine transport and the cross-linking of spectrin (Tables I and II) favours the latter explanation. There are no indications that spectrin is directly involved in carrier-mediated transmembrane transport, but it plays an essential role in the maintainance of membrane structure [25—27].

It seems likely that the augmentation of non-specific glycerol and thiourea transport is caused by photodynamic damage of the membrane structure. These two substrates have an ether/water partition coefficient (k_{ether}) of 0.00066 and 0.0063, respectively [16]. It has been suggested by Sha'afi et al. [28] that solutes with a $k_{ether} > 0.003$ would permeate in red blood cells by dissolution in the membrane, whereas solutes with a $k_{ether} < 0.003$ would cross the membrane through an aqueous path. If this were true a different influence of the photodynamic process on glycerol and thiourea permeation might have been possible. This was not borne out experimentally, however, as the increase in permeability for these two solutions was about equal.

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