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PHOTODYNAMIC EFFECTS OF HEMATOPORPHYRIN-DERIVATIVE ON TRANSMEMBRANE TRANSPORT SYSTEMS OF MURINE L929 FIBROBLASTS

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Photodynamic treatment of murine L929 fibroblasts with hematoporphyrin-derivative causes deterioration of various membrane functions. Most sensitive to photodynamic inactivation are the energy-coupled transport systems for aminoisobutyric acid and for Rb^+. The facilitated diffusion system for 2-deoxy-D-glucose is slightly less sensitive. After longer illumination periods also the membrane barrier function is impaired, as reflected by K^+ leakage and increased passive Rb^+ uptake. After still longer illumination periods intermolecular protein crosslinking can be observed. This makes it unlikely that intermolecular protein crosslinking is causally involved in the deterioration of these membrane functions.

Introduction

Activation of photosensitizers by light results in photooxidation of proteins, unsaturated fatty acids, cholesterol and nucleic acids [1–3].

Secondary reactions of photoproducts cause protein crosslinking and DNA-protein crosslinking [3].

A well-known class of sensitizers are porphyrins. Previous studies have shown that illumination of red blood cells in the presence of protoporphyrin, a clinically important representative of this class [4], results in disturbances of various membrane functions. This is reflected by a decrease of enzyme activities [1,5], decreased membrane deformability [6] and impaired transport activities [7]. The increased cation permeability, ultimately leading to photohemolysis [8], is caused by photooxidation of membrane proteins, rather than by crosslinking of membrane proteins [9].

Photoradiation therapy is a recently introduced treatment of malignant tumors [10], utilizing hematoporphyrin-derivative as photosensitizer. Hematoporphyrin-derivative is a mixture of porphyrins obtained by acetic acid/sulfuric acid treatment of hematoporphyrin [11]. One or more yet unknown component(s) of this mixture accumulate in tumors to a higher degree than in normal tissue [12]. This phenomenon causes the rather selective killing of tumor cells by subsequent exposure to visible light.

The actual cellular target for photodynamic cell inactivation by hematoporphyrin-derivative has not yet been identified. It has been shown that membrane damage, enzyme inhibition, DNA-strandbreaks and possibly DNA-protein crosslinking are induced [13–15]. In studies, designed to explore the different possibilities of cell inactivation systematically, the photodynamic action of hematoporphyrin-derivative on some membrane functions of murine L929 cells was investigated. The results of these studies are presented in this communication.

Materials

NCTC mouse fibroblasts, clone L929, ATCC number CCL1, tissue culture products and newborn bovine serum were obtained from Flow Laboratories. Hematoporphyrin-derivative was purchased from Oncology Research and Development Inc., Cheektowaga, NY. 2-Deoxy-D-[1-^{14}C]glucose, 2-amino[1-^{14}C]isobutyric acid and ^{86}Rb were purchased from Amersham. Liquid scintillation counting was done in Pico-Fluor 30 from Packard Instrument Company.

All other chemicals were obtained from Sigma, Baker, Merck or Difco and were of analytical quality.

Methods

L929 cells were grown in minimum essential medium (modified) with Earle's salts, 10% heat-inactivated calf serum, penicillin (50 U/ml), streptomycin (50 μg/ml) and fungizone (0.5 μg/ml) in 60×15 mm style dishes. Confluent cell layers (about 7×10^4 cells/cm^2) were incubated with 2 ml of different concentrations of hematoporphyrin-derivative in Dulbecco's phosphate-buffered saline for 1 h at 37°C in the dark. The cells were washed with Dulbecco's phosphate-buffered saline and illuminated in the same buffer with a slide projector equipped with a 150 W quartz halogen light bulb. The light beam was reflected through the bottom of the culture dish by a mirror, after having passed a filter with a sharp cut-off at 590 nm. Illumination was thus carried out with red light (instead of the much more effective white or violet light), to mimic the procedure of (clinical) phototherapy of tumors as closely as possible. In phototherapy red light is used because of the much better spectral transmittance of tissues for light of higher wavelengths. The culture dish was placed at a distance of 38 cm from the light source and evenly illuminated with a light intensity of 4 mW/cm^2. Prior to permeability experiments the cells were kept in the dark at room temperature for 5 min.

The K^+ content of the cells was determined as described by Bader et al. [16]. After illumination the cell layer was washed two times with 150 mM choline chloride (pH 7.4) and once with 1 mM Tris-HCl (pH 7.4). The cells were scraped into H_2O and HCl was added to a final concentration of 0.1 M. The suspension was dried at 85°C, 1 ml H_2O was added to the dried residue and K^+ was determined with a flame spectrophotometer. This method yielded the same K^+ values as measured after destruction of the organic material with $HClO_4$ at 180°C. Uptake of 2-deoxy-D-glucose, 2-aminoisobutyric acid and Rb^+ was determined by incubating the cell layer for 2 min with 2 ml 2.5 μM substrate in Dulbecco's phosphate-buffered saline at room temperature on a shaker in total darkness. Passive Rb^+ influx was measured by inhibiting the active influx with ouabain (1 mM final concentration). Uptake was stopped by washing the cell layer three times with 5 ml ice cold Dulbecco's phosphate-buffered saline. The cells were scraped into 2% SDS/10 mM sodium phosphate (pH 7) and dissolved by incubation at 100°C for 4 min. An aliquot of this solution was analyzed for radioactivity by liquid scintillation counting. Protein was determined by the method of Lowry et al. [17] with bovine serum albumine as a standard.

Hexokinase activity was determined according to Bergmeyer et al. [18] in the presence of 1% Triton X-100.

SDS-polyacrylamide electrophoresis was performed as described by Studier [19] with slight modifications [20]. The proteins in the gels were fixed with 50% trichloroacetic acid and subsequently stained by incubation with 0.25% Coomassie blue R 250 in 50% trichloroacetic acid. The detection limit of this procedure is about 1 μg protein, depending on the nature of the protein.

The ATP content of the cells was determined immediately after illumination with a Boehringer-Mannheim bioluminescence CLS kit in the presence of 0.5% Triton X-100.

Results

The influence of illumination on the K^+ content of the hematoporphyrin-derivative-treated L929 cells is shown in Fig. 1. The initial K^+ content amounted to 0.97 μmol/mg protein, a value in close agreement with that found by Chen et al. [21]. Illumination without prior incubation with hematoporphyrin-derivative or incubation

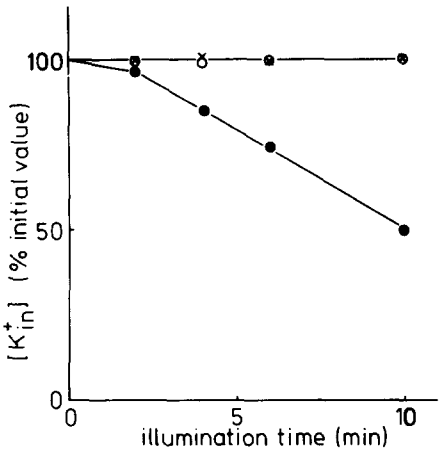

Fig. 1. K^+ content of hematoporphyrin-derivative-treated L929 fibroblasts as a function of illumination time. Preincubation with 5 μg/ml (× ——— ×), 10 μg/ml (○ ——— ○) and 25 μg/ml (● ——— ●).

with 25 μg/ml hematoporphyrin-derivative in the dark did not cause any K^+ release to the medium. Also, when incubated with 5 or 10 μg hematoporphyrin-derivative per ml, no K^+ leakage was observed within 10 min of illumination. After incubation with 25 μg hematoporphyrin-derivative per ml, however, a progressive K^+ leakage occurs upon illumination.

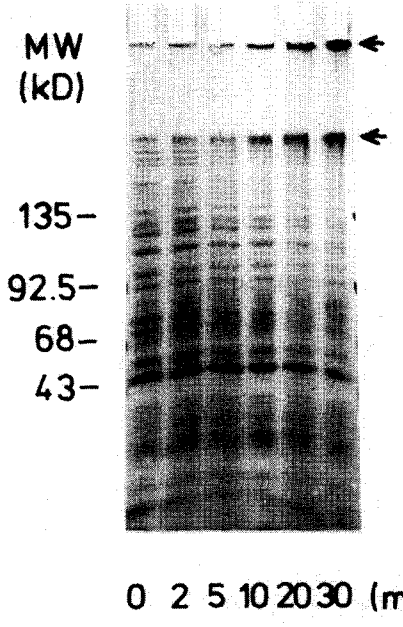

Fig. 2. Intermolecular crosslinking of total cellular proteins after preincubation with 25 μg/ml of hematoporphyrin-derivative and subsequent illumination, as shown by SDS-polyacrylamide gel electrophoresis. Arrows, crosslinked protein aggregates.

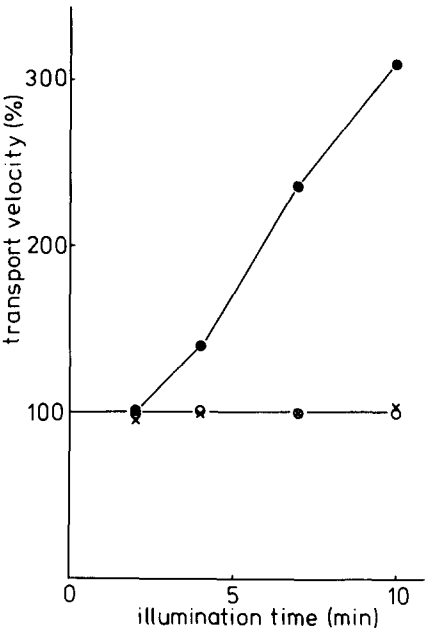

Fig. 3. Passive uptake of Rb^+, expressed as percent of initial transport velocity (100% corresponds to 8.6 pmol/min per mg protein), after preincubation with hematoporphyrin-derivative and subsequent illumination. Active Rb^+ uptake was inhibited by addition of 1 mM ouabain. For symbols, see legend to Fig. 1.

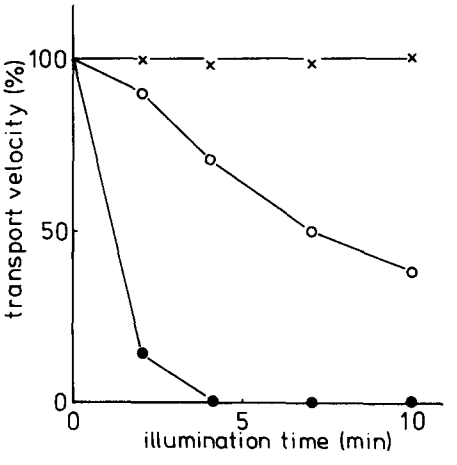

Fig. 4. Photodynamic effect of hematoporphyrin-derivative on active Rb^+ transport into L929 cells after preincubation with different hematoporphyrin-derivative concentrations. Initial transport velocity: 18.8 pmol/min per mg protein. For symbols, see legend to Fig. 1.

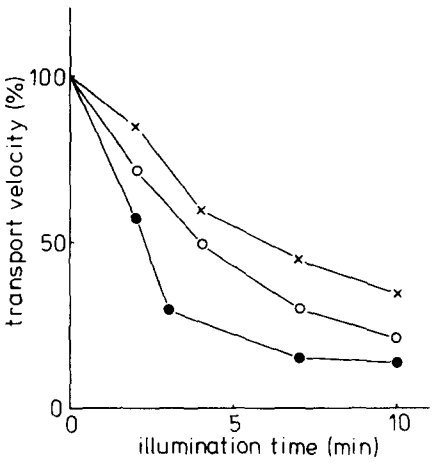

Fig. 5. Hematoporphyrin-derivative-induced photodynamic inhibition of 2-aminoisobutyric acid uptake. Initial transport velocity: 5.1 pmol/min per mg protein. For symbols, see legend to Fig. 1.

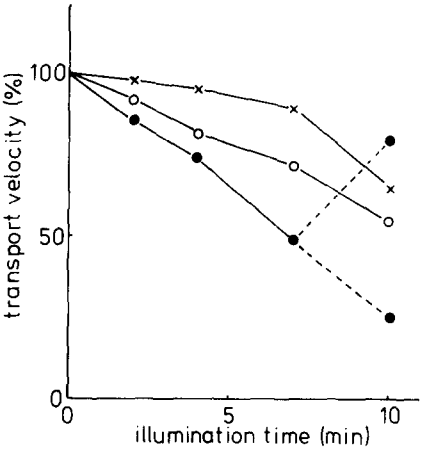

Fig. 6. Percent of initial transport velocity of 2-deoxy-D-glucose uptake as a function of illumination time after preincubation with hematoporphyrin-derivative. Initial transport velocity: 8.3 pmol/min per mg protein. For symbols, see legend to Fig. 1.

The first indications of intermolecular crosslinking of proteins could be detected in cells, pretreated with 25 μg hematoporphyrin-derivative per ml, after an illumination period of 10 min (Fig. 2). Crosslinking is reflected by the appearance of high molecular weight protein aggregates on top of the gels. As judged from K^+ leakage (Fig. 1), considerable damage of the membrane barrier function had occurred already before that point of time.

Both active and passive (ouabain-insensitive) Rb^+ uptake were linear as a function of time, at least up to 60 min. The passive Rb^+ influx did not change after illumination when the cells were incubated with 5 or 10 μg hematoporphyrin-derivative per ml, but was strongly increased, using a concentration of 25 μg/ml (Fig. 3). In Fig. 4 the photodynamic effect on active Rb^+ influx is depicted. Both after preincubation with 10 and 25 μg hematoporphyrin-derivative per ml, illumination caused a progressive inhibition.

2-Aminoisobutyric acid influx was linear up to about 40 min. This substrate accumulated to a level of about 0.32 nmol/mg protein. Taking into account a cellular volume of $22 \cdot 10^{-7}$ μl [22] and a protein concentration of 0.25 mg/10^6 cells, this means an accumulation ratio of 14.5, indicating that this transport must be energy-dependent. The results, shown in Fig. 5, show that even at the lowest hematoporphyrin-derivative concentration 2-aminoisobutyric acid influx is inhibited by subsequent illumination.

2-Deoxy-D-glucose uptake was linear for about 60 min. Illumination caused inhibition at all hematoporphyrin concentrations studied. In some experiments uptake of 2-deoxy-D-glucose was strongly inhibited after 10 min of illumination at 25 μg hematoporphyrin-derivative per ml, whereas in other experiments uptake was apparently increased (Fig. 6, dashed lines). This latter phenomenon was presumably caused by a breakdown of

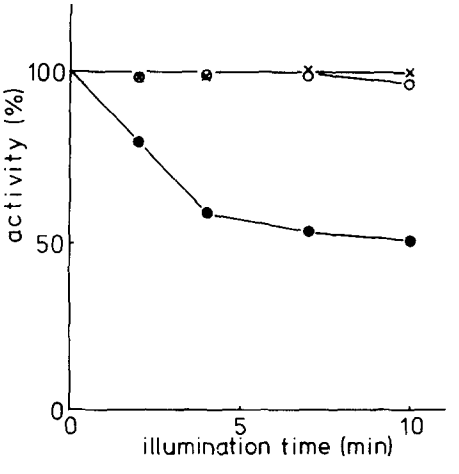

Fig. 7. Photodynamic inhibition of hexokinase enzyme activity after preincubation with hematoporphyrin-derivative. For symbols, see legend to Fig. 1.

the diffusion barrier, allowing increased influx by diffusion.

As 2-deoxy-D-glucose is phosphorylated intracellularly, hexokinase activity was also determined. Only after incubation of the cells with 25 μg hematoporphyrin-derivative per ml, photodynamic inhibition of enzyme activity could be observed (Fig. 7).

The intracellular ATP concentration amounted to 1 μM. Incubation with hematoporphyrin-derivative up to 25 μg/ml and subsequent illumination up to 10 min caused no alteration of the ATP concentration.

Discussion

Most authors tend to ascribe photodynamic cell inactivation to membrane damage, rather than to other possible mechanisms [23,24]. Therefore, hematoporphyrin-derivative-induced photodynamic effects on some crucial membrane functions were measured, in the context of a systematic study of photodynamic inactivation of L929 cells. In all experiments cells were incubated with three different hematoporphyrin-derivative concentrations for 1 h at 37°C and subsequently illuminated.

As pointed out in the introduction hematoporphyrin-derivative consists of several components. The results presented in this paper are caused by the (unknown) component(s) that are taken up by the cells during the 1 h incubations. Therefore they do not permit any conclusions concerning the sites of action, affinity for cellular loci or phototoxicity of the individual porphyrins.

Photodynamic action after incubation with the lowest two hematoporphyrin-derivative concentrations did not provoke K^+ leakage, nor increased passive Rb^+ uptake. This indicates that the membrane barrier with respect to these cations remained intact. Only after preincubation with 25 μg hematoporphyrin per ml a progressively increased leakage of K^+ and Rb^+ is observed (Figs. 1 and 3). As discussed in detail for red blood cells [8], this increased passive permeability of cations will ultimately lead to osmotic swelling and lysis.

As compared to passive, ouabaine-insensitive Rb^+ uptake, active Rb^+ influx (occurring via the $(Na^+ + K^+)$-dependent ATPase) is much more sensitive to the photodynamic action of hematoporphyrin-derivative. Even after preincubation with 10 μg hematoporphyrin-derivative per ml a progressive inhibition of active Rb^+ influx after illumination is observed (Fig. 4).

In most mammalian cells 2-aminoisobutyric acid is predominantly transported by the socalled A system for amino acids [25], which is an Na^+-dependent active transport system [25]. In L929 cells an accumulation ratio of 14.5 was observed, in accordance with the energy-linked character of this transport system. After preincubation with 10 or 25 μg hematoporphyrin-derivative per ml the sensitivity to photodynamic inactivation of this transport system is roughly comparable to the sensitivity of the active Rb^+ transport system. With 5 μg hematoporphyrin-derivative per ml, however, the situation is quite different. A rather strong inhibition of 2-aminoisobutyric acid is now observed during illumination, whereas active Rb^+ transport is not affected (Figs. 4 and 5).

2-Deoxy-D-glucose is transported by a facilitated diffusion process [26] and subsequently phosphorylated inside the cells by hexokinase. The inhibition of 2-deoxy-D-glucose uptake after photodynamic treatment can not be attributed to decreased trapping of 2-deoxy-D-glucose phosphate inside the cells, as the hexokinase activity is rather insensitive to photodynamic inactivation (Fig. 7). Therefore the data depicted in Fig. 6 actually reflect inhibition of the 2-deoxy-D-glucose transport system.

Deterioration of the studied membrane functions occurred at lower hematoporphyrin concentrations or shorter illumination times, than required to cause obvious protein crosslinking (Fig. 2). Therefore, the disturbances of function should presumably be ascribed to photooxidation of various targets, rather than to the secondary process of protein crosslinking. In comparable studies with red blood cells a similar conclusion was reached [7,9,27]. It can not be excluded, however, that early, more or less specific crosslinking of some (quantitatively minor) membrane proteins might escape detection. Further, intramolecular crosslinking can not be detected by electrophoresis and their possible contribution to disturbed function can therefore not be evaluated by the present procedures.

In general the experimental results indicate, that specific, carrier-mediated transport systems in L929 cells are more sensitive to photodynamic inactivation than the membrane barrier function. In all three carrier systems studied, illumination after preincubation with 10 μg hematoporphyrin-derivative per ml caused transport inhibition, whereas passive K^+ and Rb^+ movements were not affected under these conditions. Photoinactivation of the energy-dependent transport systems (viz. for Rb^+ and for 2-aminoisobutyric acid) could result either from deterioration of the energy yielding machinery of the cells or from photooxidation of susceptible amino acid residues in the carrier proteins. The insensibility of the intracellular ATP pool to photodynamic action is in favor of the latter explanation.

It is clear that the shape of the inhibition curves for the three specific transport systems are rather different. These differences appeared to be highly reproducible and may, at least partly, be related to differences in the mechanism of inactivation. For instance, the actual shape of an inactivation curve will depend on the number of photooxidative hits, required to destroy a particular system in a functional sense. However, as discussed (e.g., by McNally [28] in detail) curve fitting to equations, derived from particular models, will never establish the validity of a particular mechanism of inactivation, in the absence of other evidence [28]. Further studies will be necessary to elucidate these mechanisms of photodynamic inactivation at the molecular level.

Nevertheless, when comparing Figs. 4, 5 and 6, one fact is quite obvious: the relative sensitivities of the three transport systems to photodynamic inactivation depend on the hematoporphyrin-derivative concentration. At 5 μg/ml the 2-aminoisobutyric acid transport system exhibits the highest sensitivity, whereas active Rb^+ transport is not inhibited at all. At 25 μg/ml, however, the Rb^+ transport system exhibits the highest sensitivity. A possible explanation for this apparent inconsistency depends on the distribution of the sensitizer in the membrane. As porphyrin-induced photodynamic damage is mediated by the short-living singlet oxygen species [29], the actual distance between sensitizer and target molecule is of utmost importance. Assuming that the 2-aminoisobutyric acid transport system (or its direct environment) has a higher affinity for the sensitizer than for example the active Rb^+ transport system, this will contribute to the higher sensitivity of the 2-aminoisobutyric acid transport system to photodynamic inactivation at low hematoporphyrin-derivative concentrations. At higher hematoporphyrin-derivative concentrations, however, also binding to low-affinity regions (e.g., the active Rb^+ transport system) will become significant and the strong inhibition of active Rb^+ uptake now reflects the high sensitivity of this transport system for photodynamic inactivation in a more restricted sense. If this interpretation is valid, other sensitizers, with a different sequence of affinities for various membrane regions, may yield quite different results in this respect.

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