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Merocyanine 540-sensitized Photoinactivation of Soluble and Membrane-bound Enzymes in L1210 Leukemia Cells

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ABSTRACT

Merocyanine 540 (MC 540) is a photosensitizing dye that is used clinically for the purging of autologous bone marrow grafts and preclinically for the inactivation of enveloped viruses in blood products. Its mechanism of action is not yet well understood. This paper investigates the sites of MC 540-mediated photodamages in L1210 leukemia cells by examining the effects of MC 540-sensitized photoirradiation on several soluble and membrane-bound marker enzymes. When exposed to MC 540 and white light under a standard set of conditions, the activities of Na+/K+-ATPase, Mg2+-ATPase, and 5'-nucleotidase (three plasma membrane-bound enzymes) were reduced by 54, 49, and 55%, respectively. None of the intracellular enzymes included in this survey was affected by MC 540-sensitized photoirradiation as long as the plasma membrane remained intact. The two soluble enzymes, lactate dehydrogenase and malate dehydrogenase, remained refractory to MC 540-sensitized photoirradiation even after the plasma membrane had been disrupted. By contrast, the activities of the membrane-bound enzymes, NADPH-cytochrome c reductase and succinate dehydrogenase, were reduced in cell lysates by 55 and 81%, respectively. Purified NADPH-cytochrome c reductase was about 3 times less sensitive than the microsomal enzyme, suggesting that the membrane environment facilitated photoinactivation. The MC 540-sensitized photoinactivation of enzymes was accelerated in the presence of deuterium oxide and inhibited if oxygen in the medium was displaced by nitrogen or azide was added to the medium. Taken together, these data support the view that the plasma membrane is a major target of MC 540-mediated photodamages, that the inactivation of membrane-bound enzymes is an oxidative process, and that at least some photodynamic damages are mediated by type II chemistry.

INTRODUCTION

Merocyanine 540 is a photosensitizer used for the inactivation of occult leukemia, lymphoma, and neuroblastoma cells in autologous bone marrow grafts (1-7). When used under conditions that spare plumpotent hematopoietic stem cells and several therapeutically important blood products, MC 540-sensitized photoirradiation also inactivates a wide range of enveloped viruses (e.g., herpes simplex virus type 1, cytomegalovirus, human T-cell leukemia/lymphoma virus type 1, human immunodeficiency virus type 1, and Friend erythroleukemia virus) and virus-infected cells (8-11). It is conceivable that the virucidal activity of MC 540 could be exploited for the sterilization of blood products, for the ex vivo treatment of allogeneic marrow grafts from virus-positive donors prior to infusion into virus-negative recipients, or for the extracorporeal purging of autologous marrow grafts from human immunodeficiency virus-positive lymphoma patients (12) or human immunodeficiency virus-positive individuals in general.

Although MC 540 is already used clinically, its mechanism of action is still poorly understood. In an attempt to identify major cellular targets of MC 540-mediated photodynamic damages, we examined the effects of MC 540-sensitized photoirradiation on a panel of seven soluble and membrane-bound marker enzymes in L1210 leukemia cells.

MATERIALS AND METHODS

Materials. MC 540 was obtained from Kodak (Rochester, NY), fetal bovine serum from Irvine Scientific (Santa Ana, CA), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid from Research Organics (Cleveland, OH), methylcellulose (4000 cPs) from Fluka (Buchs, Switzerland), and L1210 murine leukemia cells (ATCC CCL 219) from the American Type Culture Collection (Rockville, MD). Pig liver microsomes, isolated according to Cinti et al. (13) and Yasukochi and Masters (14), and NADPH-cytochrome c reductase (NADPH:ferricytochrome oxidoreductase, EC 1.6.2.4), purified from pig liver microsomes according to Ottos et al. (15), were generous gifts from Dr. Bettie Sue Masters. Specific activities of purified preparations of NADPH-cytochrome c reductase were between 9 and 28 μmol cytochrome c reduced/min/mg protein. Turnover numbers were between 950 and 1200 nmol cytochrome c reduced/min/mmol flavin. All other reagents used in this study were from Sigma (St. Louis, MO).

Cells and Cell Lysates. L1210 cells were grown in α-medium supplemented with 10% fetal bovine serum. Cells were harvested in exponential growth phase and suspended at a density of 107/ml in N-2 hydroxyethylpiperazine-N'-2-ethanesulfonic acid-buffered (10 mM, pH 7.4) α-medium supplemented with 12% of a selected lot of fetal bovine serum. To prepare cell lysates, 15- to 20-ml aliquots of the suspension were sonicated in a VibraCell sonicator (Sonics and Materials, Inc., Danbury, CT) for 30 s. About 95% of cells were lysed by this treatment as judged by microscopic analysis. Selected experiments were duplicated by using cells that had been lysed by 3 freeze/thaw cycles, by 30 rapid passages through a 25-gauge needle, or by homogenization in a Dounce homogenizer. The mode of homogenization appeared to have no effect on initial enzyme activities or the response of enzymes to MC 540-sensitized photoirradiation.

Dye-sensitized Photoirradiation. The MC 540-sensitized photoirradiation of cells and cell lysates was performed as described previously (1, 6, 16). In brief, cells were suspended at a density of 107/ml in α-medium supplemented with 12% fetal bovine serum. Dye was added to the cell suspension or the cell lysate from a 1- mg/ml stock solution in 50% ethanol to a final concentration of 15 μg/ml, and the cell suspension or cell lysate was exposed to cool white fluorescent light (fluence rate at sample site approximately 70 W/m²) for graded time intervals. Cell suspensions or cell lysates that were exposed (a) to dye in the dark, (b) to light in the presence of the appropriate amount of vehicle (50% ethanol), or (c) to neither dye, nor light, nor solvent, served as controls. Unless stated otherwise, all three controls gave results that differed by <10%.

In Vitro Clonal Assays. In vitro clonal assays were performed as described previously (1, 16). The mean plating efficiency of samples exposed to dye in the dark or to light in the presence of the appropriate amount of vehicle was 82%. The corresponding mean colony count was 334 colonies/culture dish.

Enzyme Assays. Enzyme assays were performed immediately after completion of the photoirradiation step. The activities of Na+/K+-ATPase (ATP phosphorylase, EC 3.6.1.3.) and Mg2+-ATPase (adenosinetriphosphatase, Mg2+-activated, EC 3.6.1.4.) were determined.

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3. The abbreviations used are: MC 540, merocyanine 540; α-medium, α-modified minimal essential medium.
MC 540-SENSITIZED PHOTONACTIVATION OF ENZYMES

by quantifying the liberation of phosphate from ATP (17, 18). Na⁺/K⁺-ATPase was assayed as ouabain-inhibitable ATPase activity. Mg⁺⁺-ATPase was assayed as ouabain-insensitive activity that was stimulated by Mg⁺⁺ and ATP. All reaction mixtures were incubated at 37°C for 60 min in a reciprocating water bath. The reactions were terminated by the addition of 0.5 N HCl, and the liberation of phosphate was quantified spectrophotometrically (17, 19).

Measurements of the activity of 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) were based on the spectrophotometric determination of phosphate liberated from 5'-AMP. Assay mixtures were prepared and incubated as described for the assay of ATPases, except that the substrate buffer consisted of 100 mM glycine (pH 8.5), 10 mM MgCl₂, and 5 mM 5'-AMP (20), and that the incubation time was only 30 min.

Malate dehydrogenase (l-malate:NAD oxidoreductase, EC 1.1.1.37) was assayed according to the method of Englund and Siegel (21). Succinate dehydrogenase (succinate:cytochrome c oxidoreductase, EC 1.3.99.1) was assayed according to the method of Tsai et al. (20) and lactate dehydrogenase (l-lactate:NAD oxidoreductase, EC 1.1.1.27) according to the method of Moldeus et al. (22). NADPH-cytochrome c reductase from L1210 cells was measured as described by Sottocasa et al. (23), and NADPH-cytochrome c reductase from porcine liver as described by Masters et al. (24).

Protein concentrations were determined by the method of Lowry et al. (25), using bovine serum albumin as a standard. To assess the role of oxygen in the inactivation of enzymes, some samples were depleted of oxygen by gently bubbling nitrogen through the cell suspensions/cell lysates for 30 s immediately before the irradiation step.

Oxygen Measurements. Oxygen measurements were performed with a Model ABL3 blood gas analyzer (Radiometer, Copenhagen, Denmark).

RESULTS

Inactivation of in Vitro Clonogenic Cells and Permeability of Plasma Membranes. Simultaneous exposure of L1210 cells to MC 540 under standard conditions for 75 min reduced the concentration of in vitro clonogenic cells by >5 log (Fig. 1). However, only about 20% of the intracellular content of lactate dehydrogenase was released into the supernatant (Fig. 1). This indicated that the plasma membrane of the majority of cells had not yet become permeable to macromolecules, despite the fact that the cells had sustained damages that eventually would prove lethal for >99.999% of the population.

Inactivation of Plasma Membrane-bound Enzymes. MC 540-sensitized photoirradiation reduced the activity of all three plasma membrane-bound enzymes, Na⁺/K⁺-ATPase (Fig. 2), Mg⁺⁺-ATPase (not shown), and 5'-nucleotidase (Fig. 3). When intact L1210 cells were exposed to MC 540 and light for 75 min (315 kJ/m²) under standard conditions, enzyme activities were reduced by 54, 49, and 55%, respectively. Rates of inactivation were similar for enzymes in intact cells and for enzymes in cell lysates (Figs. 2 and 3). Whereas survival curves of clonogenic cells always showed a pronounced shoulder (Fig. 1; Refs. 1–3 and 6), no lag phase was evident in the inactivation of plasma membrane-bound enzymes (Figs. 2 and 3).

Inactivation of Soluble Enzymes. When intact cells were exposed to MC 540 and light, the cytoplasmic enzyme, lactate dehydrogenase, was not affected (data not shown). The mitochondrial matrix enzyme, malate dehydrogenase, was completely refractory to MC 540-sensitized photoirradiation, whether it was exposed to dye and light in the form of intact cells or in the form of a cell lysate (data not shown).

Inactivation of Membrane-bound Mitochondrial and Microsomal Enzymes. MC 540-sensitized photoirradiation of intact
cells had no effect on the activity of the mitochondrial enzyme, succinate dehydrogenase (Fig. 4). However, in cell lysates, the enzyme was rapidly inactivated (Figs. 4 and 6). Similarly, NADPH-cytochrome c reductase in intact L1210 cells was refractory to MC 540-sensitized photoirradiation, whereas in cell lysates, the same enzyme was inactivated (Fig. 5), although not as rapidly as succinate dehydrogenase.

Experiments conducted with different preparations of NADPH-cytochrome c reductase indicated that the membranous environment facilitated the inactivation of the enzyme by MC 540-sensitized photoirradiation. Enzyme presented as a (membrane-bound) microsomal fraction was inactivated with a half-life of 22 min (92 kJ/m2) (Fig. 5). By contrast, the detergent-purified and the proteolytically cleaved preparations were inactivated much more slowly (Fig. 5). In accordance with earlier reports on the photosensitivity of flavin-containing enzymes (26), purified NADPH-cytochrome c reductase was sensitive to photoirradiation by white light in the absence of dye (Fig. 5). After 75 min (315 kJ/m2) of irradiation (no dye), the activities of the protease-cleaved and the detergent-solubilized forms were reduced by 15 and 32%, respectively (data not shown). By contrast, crude lysates of L1210 cells and pig liver microsomes showed no loss of enzyme activity due to photoirradiation alone (data not shown).

Role of Oxygen and Singlet Oxygen in MC 540-mediated Photoinactivation of Enzymes. The MC 540-sensitized photo-inactivation of enzymes was oxygen dependent. Both 5'-nucleotidase, a plasma membrane-bound enzyme, and succinate dehydrogenase, a mitochondrial enzyme, were protected against MC 540-sensitized photoirradiation if 80% (Table 1) of the oxygen in the incubation medium was displaced by nitrogen (Figs. 3 and 4). As Table 1 indicates, very little oxygen entered the vessel during the 75-min irradiation period.

Replacing 87% of the water in the incubation medium with deuterium oxide (which is known to increase the half-life of singlet oxygen approximately 5-fold (27, 28), accelerated the dye-sensitized photoinactivation of enzymes (Figs. 3–5). Azide, a known singlet oxygen scavenger, protected succinate dehydrogenase from dye-sensitized photoinactivation while mannitol had almost no protective effect (Fig. 6).

DISCUSSION

Due to its negative charge, MC 540 does not readily penetrate intact cells (29). Direct microscopic observations show that...
during the initial phase of the dye-sensitized photoirradiation of electrically excitable and leukemic cells, MC 540 binds primarily to the plasma membrane (29, 30). Significant binding of dye to intracellular structures is only detectable after exposure to high doses of dye and light that are sufficient to kill the majority of cells. Photoexcited MC 540 generates singlet oxygen (31–33), and there is growing evidence that singlet oxygen is one (but not necessarily the only) mediator of its cytotoxic and antiviral activity (11, 16, 31). Since singlet oxygen is very reactive and short lived, it is reasonable to speculate that it exerts most of its damaging effects close to where it is generated, i.e., in the plasma membrane.

The data presented in this paper are compatible with the view that the plasma membrane is the primary target of the cytotoxic effects of MC 540. The plasma membrane-bound enzymes, Na+/K+-ATPase, Mg2+-ATPase, and 5′-nucleotidase, were rapidly inactivated by MC 540-sensitized photoirradiation, while the intracellular enzymes, lactate dehydrogenase, succinate dehydrogenase, malate dehydrogenase, and NAPDH-cytochrome c reductase, were not affected. As comparisons between enzyme activities in intact cells and cell lysates showed, not all intracellular enzymes were intrinsically resistant to MC 540-sensitized photoirradiation. Some were merely shielded from the dye and its toxic effects by the plasma membrane. Once the integrity of the plasma membrane was compromised, succinate dehydrogenase and NAPDH-cytochrome c reductase became susceptible to MC 540-sensitized photoirradiation.

Data on enzyme inhibition kinetics were indicative of a first-order process. As previously reported for the inhibition of Na+/K+-ATPase and Mg2+-ATPase in rose Bengal-sensitized cells (34) and the MC 540-mediated inhibition of transmembrane transport in L1210 cells and normal lymphocytes (35), the MC 540-sensitized photoinactivation of enzymes showed no lag phase, suggesting a single-hit mechanism. By contrast, the MC 540-sensitized photoinactivation of cells shows a pronounced lag phase that is more compatible with a multiple-hit mechanism (1–3, 6).

Displacement of oxygen in the medium by nitrogen inhibited the dye-sensitized photoinactivation of enzymes, indicating that the inactivation of enzymes was mediated by an oxidative process. Replacing 87% of the water content of the incubation medium by deuterium oxide accelerated the inactivation of enzymes, while the addition of azide, a known scavenger of singlet oxygen, inhibited it. The observed effects of deuterium oxide and azide are, by themselves, not strictly diagnostic of a type II mechanism. However, if viewed in context with the direct identification of singlet oxygen by spectrophotometric methods (33) in liposomes and the identification of 5-α-cholest-6-ene-3β,5-diol (31) in red cell membranes, they suggest that the inactivation of enzymes is at least in part mediated by type II chemistry.

The stimulation by deuterium oxide and the inhibition by azide may seem modest. However, it should be noted that all experiments were conducted in the presence of serum proteins which are known to be efficient quenchers of singlet oxygen (36), and that MC 540 localizes preferentially in hydrophobic domains of the plasma membrane to which azide and deuterium oxide may have only limited access.

In this limited survey, membrane-bound enzymes were found to be more sensitive to MC 540-sensitized photoirradiation than soluble enzymes. Furthermore, NAPDH-cytochrome c reductase as a membrane-bound enzyme was more sensitive than two solubilized forms of the same enzyme. A similar preferential inactivation of membrane-bound enzymes has been reported for cells treated with hematoporphyrin derivative (37, 38). There are several plausible explanations for the preferential inactivation of membrane-bound enzymes by MC 540-sensitized photoirradiation. (a) MC 540 is known to partition into lipid bilayers and to localize in hydrophobic domains of the plasma membrane (39, 40). This may lead to a local concentration of dye and reactive oxygen species in the immediate vicinity of membrane-bound enzymes. (b) Singlet oxygen has a longer half-life in solvents with a low dielectric constant (e.g., biological membranes) (41). Furthermore, in a lipid membrane, singlet oxygen is not hindered in its diffusion by charged or neutral water-lipid interfaces, and, therefore, is not compartmentalized (27). (c) The inactivation of membrane-bound enzymes may also be facilitated by a relatively high concentration of cysteine, methionine, tryptophan, histidine, and tyrosine residues whose rate constants for interactions with singlet oxygen are 2 to 3 orders of magnitude higher than the corresponding rate constants for unsaturated lipids (26, 42). (d) It is conceivable that MC 540, as it undergoes the light-dependent translocation into a membrane (29, 30, 39), perturbs the structure of the membrane in such a way that it impairs the function of a membrane-bound protein. MC 540-mediated perturbations of membrane order have been observed in liposomes by using differential scanning calorimetry (43). The lipid milieu is known to be important for the functional integrity of transport proteins such as ATPases (44). However, it should be noted that 5′-nucleotidase and NAPDH-cytochrome c reductase, two enzymes with relatively short membrane spanning regions, were as sensitive to MC 540-sensitized photoirradiation as ATPases.

In summary, the data presented in this paper support the view that MC 540 exerts its cytotoxic effects primarily at the level of the plasma membrane and that plasma membrane-bound enzymes that are essential for cell survival are molecular targets of MC 540-mediated photodamages.

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REFERENCES


