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Merocyanine 540-sensitized photokilling of leukemia cells: role of post-irradiation chain peroxidation of plasma membrane lipids as revealed by nitric oxide protection

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Abstract

The lipophilic dye merocyanine 540 (MC540) localizes primarily in the plasma membrane (PM) of tumor cells, where it can sensitize lethal photoperoxidative damage of potential therapeutic importance. We postulated (i) that chain peroxidation triggered by iron-catalyzed turnover of nascent hydroperoxides (LOOHs) generated by singlet oxygen ($^1O_2$) attack on PM lipids contributes significantly to overall cytolethality, and (ii) that nitric oxide (NO), a known scavenger of organic free radicals, would suppress this and, thus, act cytoprotectively. In accordance, irradiation of MC540-sensitized L1210 cells produced 5α-OOH, a definitive $^1O_2$ adduct of PM cholesterol, which decayed during subsequent dark incubation with appearance of other signature peroxides, viz. free-radical-derived 7α/β-OOH. Whereas chemical donor (SPNO or SNAP)-derived NO had little or no effect on post-irradiation 5α-OOH disappearance, it dose-dependently inhibited 7α/β-OOH accumulation, consistent with interception of chain-carrying radicals arising from one-electron reduction of primary LOOHs. Using [^{14}C]cholesterol as an L1210 PM probe, we detected additional after-light products of chain peroxidation, including diols (7α-OH, 7β-OH) and 5,6-epoxides, the yields of which were enhanced by iron supplementation, but strongly suppressed by NO. Correspondingly, photoinitiated cell killing was significantly inhibited by NO introduced either immediately before or after light exposure. These findings indicate that prooxidant LOOH turnover plays an important role in photokilling and that NO, by intercepting propagating radicals, can significantly enhance cellular resistance.

Keywords: Merocyanine 540; Photosensitization; Lipid peroxidation; Nitric oxide; Free radical

1. Introduction

Many natural pigments and synthetic dyes are being evaluated for therapeutic purposes, e.g., as photosensitizing agents in antitumor photodynamic therapy (PDT) [1,2]. Among these, the anionic amphiphile merocyanine 540 (MC540) has elicited special interest in connection with its ability to selectively inactivate neoplastic cells (leukemia, lymphoma, neuroblastoma cells) in autologous bone marrow grafts [3] and also pathogenic viruses in bone marrow and blood fractions [4]. Singlet oxygen ($^1O_2$) has been reported to be an important intermediate in these photodynamic effects [5]. MC540 localizes mainly in the plasma

Abbreviations: BHT, butylated hydroxytoluene; BSA, bovine serum albumin; Ch, cholesterol; ChOOH(s), cholesterol hydroperoxide(s); ChOX, cholesterol oxide(s); DFO, desferrioxamine; $Fe(HQ)_3$, ferric-8-hydroxyquinoline; MC540, merycyanine 540; HPLC-EC(Hg), high-performance liquid chromatography with mercury cathode electrochemical detection; HPTLC-PI, high-performance thin layer chromatography with phosphorimaging detection; LOOH(s), lipid hydroperoxide(s); PDT, photodynamic therapy; SNAP, *S*-nitrosyl-*N*-acetyl-D,L-penicillamine; SPNO, (*Z*)-1-{*N*-(3-aminopropyl)-*N*-[4-(3-aminopropylammonio)butyl]amino}diazen-1-ium-1,2-diolate (spermine NONOate); 5α-OOH, 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide; 5α-OH, 5α-cholest-6-ene-3β,5-diol; 6α(β)-OOH, 3β-hydroxycholest-4-ene-6α(β)-hydroperoxide; 6α(β)-OH, cholest-4-ene-3β,6α(β)-diol; 7α(β)-OOH, 3β-hydroxycholest-5-ene-7α(β)-hydroperoxide; 7α(β)-OH, cholest-5-ene-3β,7α(β)-diol; 5,6-epoxide, cholestan-5,6-epoxy-3β-ol

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membrane of tumor cells [1,6,7] and most of its phototoxicity is attributed to damage occurring in this compartment [1]. Studies on plasma membrane models (liposome mimetics, erythrocyte ghosts) have shown that unsaturated phospholipids and cholesterol are readily peroxidized during MC540/light treatment [5,8,9], the initial products of $^1O_2$ attack being lipid hydroperoxide (LOOH) species. Higher-polarity LOOHs might be deleterious to membrane structure/function in and of themselves, or might serve as initiators of iron-catalyzed free radical (chain) reactions that are even more detrimental [10,11].

Nitric oxide (NO) generated naturally via the nitric oxide synthase-catalyzed 5-electron oxidation of L-arginine is involved in numerous normo- and pathophysiological processes ranging from vasodilation and immunodefense to atherogenesis and carcinogenesis [12]. NO itself is not an effective oxidant, but can be converted to strong damaging oxidants such as peroxynitrite and nitrogen dioxide under biological conditions [13]. On the other hand, NO on its own can act as a potent lipid antioxidant, e.g., by scavenging chain propagating oxyl or peroxyl radicals, and its ability to partition into membrane bilayers favors this activity [14–16]. Interception of lipid-derived radicals by NO could contribute to overall cellular resistance to peroxidative stress [17,18], and, if occurring during PDT-induced stress, might compromise treatment efficacy. We showed recently that photoinitiated chain peroxidation and necrotic death of protoporphyrin IX-overproducing COH-BR1 cells was strongly suppressed by exogenous NO supplied at the onset of irradiation [19,20]. Although these effects were mainly attributed to quenching of chain-carrying post-photon lipid oxyl/peroxyl radicals on the plasma membrane, the evidence for this was largely indirect. The purpose of the present study was to establish the cytotoxic importance of post-irradiation chain peroxidation, using MC540 as a plasma membrane sensitizer and exogenous NO as a free radical interceptor.

2. Materials and methods

2.1. Materials

MC540 was obtained from Eastman Kodak (Rochester, NY). RPMI-1640 growth medium, fetal bovine serum, antibiotics, fatty-acid-free bovine serum albumin (BSA), desferrioxamine (DFO), 8-hydroxyquinoline, Hoechst 33258, and unlabeled cholesterol (Ch) were obtained from Sigma (St. Louis, MO). Ferric 8-hydroxyquinoline [$Fe(HQ)_3$, 1.0 mM in 50% ethanol] was prepared as described [16]. Cayman Chemical (Ann Arbor, MI) supplied the spermine NONOate (SPNO) and *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP). Stock solutions of 25 mM SPNO in 10 mM NaOH were stored at −80 °C; the donor was stable under these conditions, but at pH 7.4 and 37 °C decayed with a half-life of ~39 min [16,21], giving spermine and two equivalents of NO. Decomposed SPNO for control experiments was prepared by acidifying a stock sample to pH 3. Stock SNAP (10 mM in dimethylsulfoxide) was prepared in subdued light immediately before addition to cells. Exposure to ambient light at room temperature for 3–4 days gave fully decomposed SNAP. [4-^{14}C]Ch (~50 mCi/mmol) was obtained from Amersham (Arlington Heights, IL). Immediately before use, the material (referred to as [^{14}C]Ch) was separated from any preexisting oxidation products as described [22]. Cholesterol hydroperoxide (ChOOH) standards for HPLC-EC(Hg), including $^1O_2$-generated 5α-OOH and free-radical-generated 7α/β-OOH, were prepared and quantified as described [23], as were the ^{14}C-labeled cholesterol oxide (ChOX) standards for HPTLC-PI, including 5α-OOH, 5α-OH, 6β-OH, 7α/β-OOH, 7α-OH, and 7β-OH [23,24]. Product assignments were confirmed by ^{1}H nuclear magnetic resonance and mass spectrometric analyses [16,22]. The 5α,6α-epoxide of Ch (also used as a standard) was obtained from Steraloids (Wilton, NH). Burdick and Jackson (Muskegan, MI) supplied the HPLC-grade solvents.

2.2. Cell culture and [^{14}C]Ch labeling

Murine lymphocytic leukemia L1210 cells obtained from the American Type Culture Collection (Rockville, MD) were cultured in 10% serum/RPMI medium supplemented with sodium selenite, insulin, transferrin, and antibiotics, as specified [25]. All experiments were carried out on exponentially growing cells. As needed, L1210 cells were labeled with [^{14}C]Ch, which served as a probe for free radical activity occurring primarily in the plasma membrane [26,27]. Cells were first incubated in the presence of 50 μM DFO overnight in order to deplete non-heme iron, which might otherwise promote Ch autoxidation during labeling. After washing with and resuspending in RPMI medium, the cells (5×10^6/ml) were labeled with [^{14}C]Ch by incubating with a [^{14}C]Ch/BSA complex (1:15 mol/mol, 4 mg BSA/ml) for 1 h at 37 °C. After treatment with BSA (5 mg/ml) to remove non-membrane-bound label, cells were washed and resuspended in RPMI to a concentration of ~10^7/ml. Other details were as described previously [26].

2.3. Cell sensitization, irradiation, NO exposure, and viability determination

Cells (~1.0×10^7/ml in RPMI without serum), either unlabeled for HPLC-EC(Hg) analyses or [^{14}C]Ch-labeled for HPTLC-PI analyses, were sensitized in 75-cm^2 flasks by incubating with 25 μM MC540 for 30 min in the dark. After removal of samples for determination of preexisting ChOX, the flasks were irradiated at room temperature on a translucent plastic platform over a twin bank of 40 W cool white fluorescent tubes [25]; fluence rate at the platform surface was ~0.6 mW/cm^2. Typical light exposure time was 20 min, equivalent to a delivered fluence of ~0.72 J/cm^2.