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Advances in Brief

Membrane Lipid Free Radicals Produced from L1210 Murine Leukemia Cells by Photofrin Photosensitization: An Electron Paramagnetic Resonance Spin Trapping Study[1]

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Abstract

We have detected membrane lipid-derived free radicals from neoplastic cells subjected to Photofrin photosensitization. The presence of the prooxidants iron or iron plus ascorbate in the L1210 cell system increased the intensity of the spin-trapped lipid radical electron paramagnetic resonance spectra and correspondingly decreased cell survival. In addition, raising the proportion of unsaturated lipids in the cell membranes by supplementation of the growth medium with docosahexaenoic acid increased lipid radical formation and decreased cell survival when the L1210 cells were subjected to Photofrin and light. These data educe the hypothesis that the extent of radical generation as well as the efficacy of photodynamic therapy can be increased when prooxidant conditions, which enhance free radical processes, are present in conjunction with photosensitizers that target membrane lipids.

Introduction

Photofrin, a partially purified preparation of hematoporphyrin derivative, is being developed for use as a photosensitizer in the PDT[3] of cancer (1–3). Photodynamic action relies on the absorption of visible light by the photosensitizer to form its excited triplet state. This excitation energy is transferred to oxygen, producing excited state singlet oxygen, $^1O_2$. This highly reactive, electrophilic oxygen species initiates oxidation processes that kill cells (1–3). The hydrophobic character of Photofrin causes it to localize in plasma and subcellular membranes, making these structures especially sensitive to photodamage (4). It has been demonstrated that singlet oxygen production during photosensitization by Photofrin (or a similar preparation of hematoporphyrin derivative) of model membrane systems, as well as cells, substantially increases their LOOH concentration (4–7). We hypothesize that these LOOHs could serve as initiating agents for "dark" free radical-mediated lipid peroxidation. Indeed, it has been recently demonstrated that murine L1210 leukemia cells and human CaSki cervical carcinoma cells that have a compromised ability to metabolize LOOHs are significantly more sensitive to photosensitizer-produced singlet oxygen (4, 6, 8). We report here the formation of lipid-derived free radicals when L1210 cells are subjected to Photofrin photosensitization.

Materials and Methods

Photofrin (porfimer sodium) was kindly provided by QLT Phototherapeutics, Inc., Vancouver, British Columbia, Canada. It was suspended in phosphate-buffered saline (pH 7.4) and frozen until immediately before use. Ascorbate was prepared as a 0.10 M stock solution of L-ascorbic acid (Aldrich Chemical Co., Milwaukee, WI) in distilled water. Concentration was verified by dilution in metal-free phosphate buffer, pH 7.4, using ϵ_{265} = 14,500 M^{-1} cm^{-1} for ascorbate (9). POBN (Sigma Chemical Co., St. Louis, MO) was prepared as a 1.0 M stock solution in distilled water immediately before use. Iron(II) was prepared as a 10.0 mM stock solution in ≈0.01 M HCl. The concentration of Fe^{2+} was verified with Ferrozine, ϵ_{562} = 27,900 M^{-1} cm^{-1} (10).

L1210 murine leukemia cells were grown in suspension culture at 37°C in medium consisting of RPMI 1640 (Grand Island Biochemical Co., Grand Island, NY) and heat-inactivated 10% fetal bovine serum (Sigma). Cells were harvested during exponential growth phase and suspended in 0.9% NaCl for all experimental manipulations. The cytotoxic effects of Photofrin were determined by trypan blue dye exclusion. L1210 cells at 2 × 10^6 cells/ml were incubated with 15 μg/ml Photofrin for 45 min at 37°C in the dark. After this incubation, designated concentrations of Fe^{2+} and ascorbate were added (in that order) and then exposed to the photosensitizing light. For light treatment a 2-ml aliquot of the cell suspension was placed in a 15-ml clear polypropylene centrifuge tube and illuminated with a Bausch & Lomb 120-V 15-W Reflector Illuminator (tungsten bulb) at a source-to-sample distance of 25 cm. The light was focused through a 5-cm layer of water to remove IR radiation; light intensity at the sample was 160 J m^{-2} s^{-1}.

We have shown previously that our cellular fatty acid modification protocol of supplementation of growth media with 22:6 results in cells that contain 23–37% 22:6 in cellular phospholipids (11–13). Unmodified L1210 cells contain less than 1% 22:6 (11, 14).

Photofrin uptake in cells was determined by fluorescence spectroscopy (15). We found no difference in Photofrin uptake between 22:6 modified cells and unmodified L1210 cells. Photofrin was present in the media in both the cytotoxicity determinations and the EPR experiments. However, parallel results were obtained when cells were washed of excess Photofrin prior to illumination. All experiments were carried out with an absolute minimum of room light.

For the EPR spin trapping experiments the Photofrin incubation and reagent addition were done as above except the sample size was 0.5 ml and the POBN was added prior to the Fe^{2+}/ascorbate addition. Immediately after reagent addition, the cell suspension was transferred to a TM EPR quartz flat cell. The flat cell was centered in a TM_{110} cavity of a Bruker ESP-300 EPR spectrometer and scans were begun. Light intensity on the sample in the EPR cavity was ≈160 J m^{-2} s^{-1} as measured with a Yellow Springs Instrument 65A radiometer with the 6551 probe, assuming the cavity grid transmits 75% of the incident light. For the experiments in which POBN radical adduct concentration was monitored *versus* time, each data point represents the signal average of ten scans of the low field doublet of the POBN/lipid-derived radical adduct (POBN/L˙) spectrum. For these points, instrument settings were: 10-G/21-s scan rate; 1.0-G modulation amplitude; 1.0 × 10^6 receiver gain; 0.33-s time constant; and 40 mW nominal power (16).

Results and Discussion

Preparations of hematoporphyrin derivative, similar to Photofrin, produce singlet oxygen in membranes (6), and this singlet oxygen can react with unsaturated membrane lipids to form lipid hydroperoxides (5); thus we hypothesized that free radical lipid peroxidation could be easily initiated in Photofrin/light-treated cellular membranes. We have

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[3] The abbreviations used are: PDT, photodynamic therapy; EPR, electron paramagnetic resonance; LOOH, lipid hydroperoxide; POBN, α-(4-pyridyl 1-oxide)-*N-tert*-butylnitrone; PUFA, polyunsaturated fatty acid; 22:6, docosahexaenoic acid.

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recently developed a new EPR spin trapping approach that allows us to detect, in real time, membrane lipid-derived free radicals from live cells, using POBN as the spin trap (17, 18). When this method for detecting lipid radicals is applied to Photofrin-treated L1210 cells, exposure to light results in the EPR spectrum shown in Fig. 1, *top*. This spectrum consists of two species: the central doublet species ($a^H = 1.8$ G) is the ascorbate free radical; the triplet of doublets is the POBN/L• spin adduct ($a^N = 15.65$ G, $a^H = 2.71$ G), (17, 18) where L• represents a lipid-derived free radical such as the ethyl or pentyl radical produced during lipid peroxidation (17–20). The central doublet of the POBN/L• EPR signal partially overlaps the ascorbate doublet signal. These results provide the first direct EPR evidence that lipid radicals are indeed formed as a result of Photofrin photosensitization of live cells.

Because iron and ascorbate, present in the experiment of Fig. 1, *top*, are prooxidants that can initiate lipid peroxidation (18, 21), additional studies were needed to demonstrate that Photofrin and light treatment do indeed enhance free radical production. Photofrin-treated cells in the absence of light, but in the presence of Fe^{2+} and ascorbate, produce low levels of lipid radical, as observed by the formation of POBN/L• (Fig. 1, *bottom*). In the absence of Photofrin, visible light alone results in the production of some POBN/L•. However, when Photofrin-treated cells are subjected to light, lipid free radical formation is significantly stimulated (8-fold over Photofrin-dark and 2-fold compared to light alone). Thus, Photofrin-treated cells produce increased levels of lipid-derived radicals when subjected to photosensitizing light.

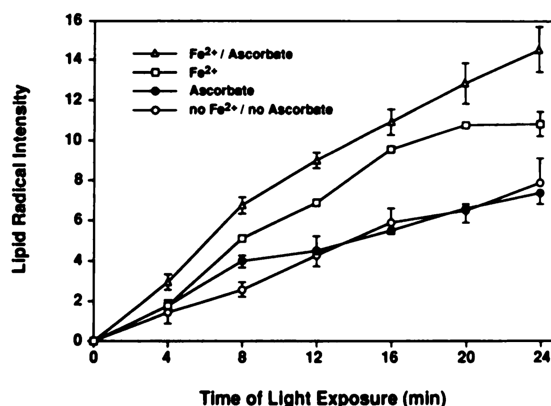

Fig. 2. Effects of the oxidative cofactors, Fe^{2+} (5 μM) and ascorbate (100 μM) on lipid radical formation from Photofrin-treated L1210 cells upon exposure to light. Because there were minor variations in the lipid radical EPR signal intensity prior to light exposure, the initial dark intensity of each sample was subtracted from the data points collected during exposure to light. Thus, we have the changes in POBN/L• *versus* time of light exposure. *Bars*, SEM of at least three replicates. No cofactors and the ascorbate only values are not statistically different at $P = 0.05$, whereas Fe^{2+} plus ascorbate is different from Fe^{2+} only, $P < 0.05$. Both curves are different from Photofrin only and ascorbate only, $P < 0.01$, using one-way analysis of covariance.

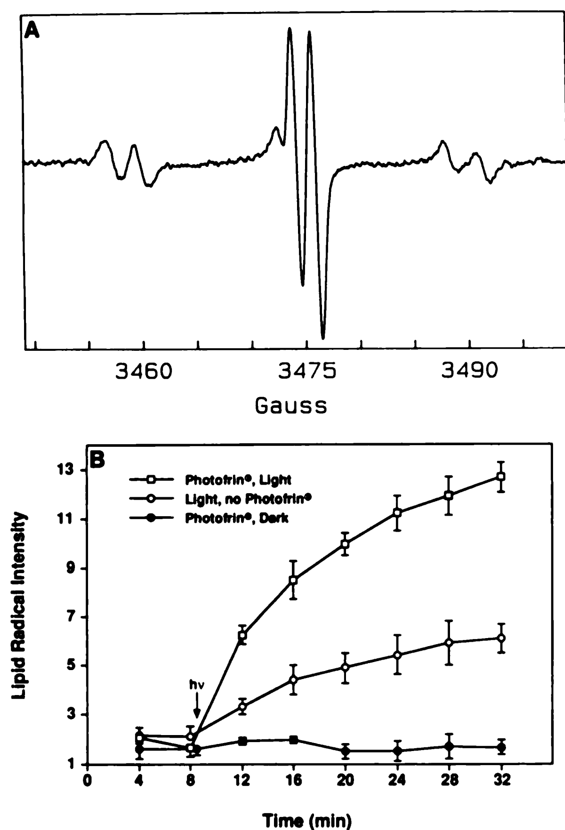

Fig. 1. Lipid radical from Photofrin and light-treated cells. *Top*, EPR spectrum showing two radical species present, POBN/L• ($a^N = 15.65$ G, $a^H = 2.71$ G) and the ascorbate radical doublet ($a^H = 1.8$ G). The solution in the TM_{110} flat cell contained Fe^{2+} (5 μM), ascorbate (100 μM), POBN (25 mM), and 2×10^6 Photofrin-treated L1210 cells/ml in 0.9% NaCl solution, pH 6.9. In the absence of Fe^{2+} and ascorbate, POBN/L• is observed, but at a much lower level (not shown). The cell suspension was illuminated directly in the EPR cavity. EPR instrument settings were: 1.0 G modulation amplitude, 1×10^6 receiver gain; 40 mW nominal power; and 60 G/336 s scan rate. This spectrum is a four-scan signal-averaged result over 22.4 min. *Bottom*, EPR signal intensity of POBN/L• produced from L1210 cells (2×10^6/ml) with Fe^{2+} (5 μM), ascorbate (100 μM), and POBN (25 mM). For the first two points of each curve there was no light exposure. Immediately after the collection of the 8 min data point (*arrow*), the light was turned on. *Bars*, SEM of at least three replicates. Before light exposure there was no significant difference between the three curves, $P > 0.15$. However, upon illumination, each of the three curves became significantly different, $P < 0.01$, typically $P < 0.005$. EPR lipid radical intensity represents the signal height of the low field doublet in arbitrary units.

The oxidative cofactors Fe^{2+} and ascorbate were both present in the experiments of Fig. 1. However, further studies indicated that iron and ascorbate have independent effects during Photofrin photosensitization of cells (Fig. 2). Ascorbate alone produced no significant change in the amount of lipid radical produced, but the addition of 5 μM Fe^{2+}, without ascorbate, significantly enhanced free radical production during Photofrin photosensitization. The presence of both iron and ascorbate synergistically enhanced lipid radical formation compared to either alone. These results demonstrate that the presence of iron is a key factor in lipid radical production from Photofrin photosensitization.

Fe^{2+} promotes lipid radical formation during Photofrin photosensitization (Fig. 2); therefore we examined the effects of the sequence of iron addition and light exposure on radical formation. In the absence of iron, Photofrin-photosensitization should result in a buildup of membrane LOOHs; the subsequent addition of Fe^{2+} to these cells would then initiate a burst of radical formation via a Fenton-type reaction,

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + OH^- + LO^\bullet$$

We also reasoned that the formation of a burst of the highly oxidizing lipid alkoxyl free radical (LO•) (20) would result in a very damaging storm of lipid peroxidation, a free radical chain reaction (20). When Fe^{2+} (with no added ascorbate) was introduced to Photofrin-treated L1210 cells just prior to light exposure, POBN/L• was observed, as expected (Fig. 3*A*). Nearly the same level of radical production was observed without light and/or without Fe^{2+} addition. Note that the light exposure was only 2 min, which is within the first time point after the beginning of light exposure in the experiments of Fig. 2. However, when Fe^{2+} is added to the cell suspension after a 2-min light exposure, a burst (≈3-fold increase) of lipid free radical formation is observed, consistent with our hypothesis.

If these free radical events are central to Photofrin-phototoxicity, then one would hypothesize that free radical formation should correlate inversely with cell survival. We conducted experiments similar to

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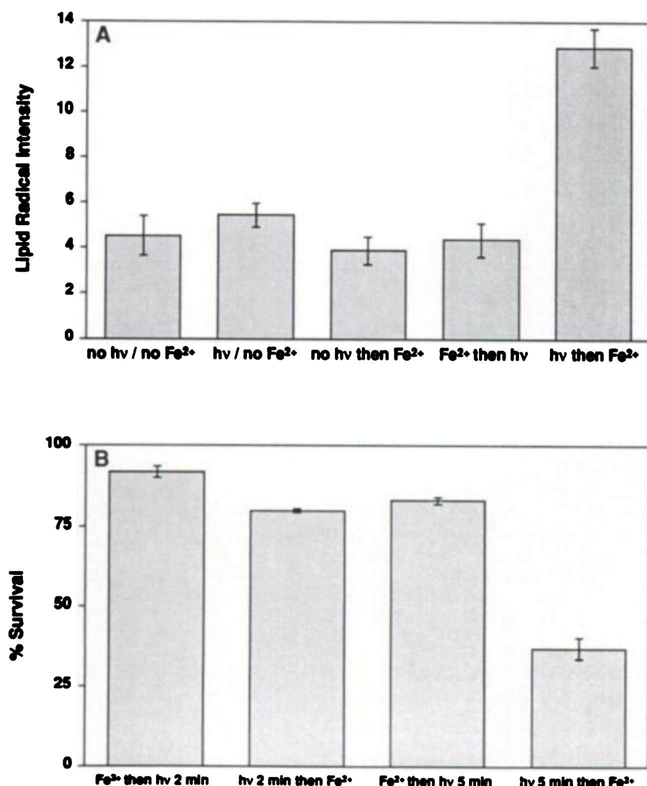

Fig. 3. Influence of iron-light sequence on radical formation and cytotoxicity. *A*, effect of the order of light exposure (*hv*) and iron addition on POBN/L· spin adduct formation, determined using Photofrin-treated L1210 cells and exposing them to light for 0 or 2 min. Fe^{2+} (5 μM, no added ascorbate) was added to the cell suspension either before or after light exposure. This short, 2-min exposure to light produced a statistically different radical intensity only when iron was added after light exposure ($P < 0.005$). For all other comparisons $P > 0.05$, which was expected from examination of Figs. 2 and 3. *B*, effect of the order of light exposure and iron addition on cytotoxicity, determined using Photofrin-treated L1210 cells and exposing them to light for 2 or 5 min. Iron (5 μM Fe^{2+}, no added ascorbate) was introduced to the cell suspension either before or after light exposure. The addition of Fe^{2+} after light exposure produced significant decreases in cell survival compared to Fe^{2+} addition before illumination ($P < 0.005$) with the greatest of these decreases being observed at 5 min of light exposure.

those in Fig. 3*A*, but rather than radical formation, immediate cytotoxicty was measured by trypan blue dye exclusion from cellular membranes. In these experiments, Fe^{2+} alone, *i.e.*, no added ascorbate, was used to initiate free radical processes. Indeed, in those experiments where Fe^{2+} was added after light exposure, the sequence that produced the most radicals in the experiments of Fig. 3*A*, we observed the largest decrease in cell survival (Fig. 3*B*). A longer 5-min light exposure resulted in an even greater decrease in cell survival than those exposed for only 2 min. These data demonstrate that the availability of Fe^{2+} after Photofrin-photosensitization has the potential to greatly increase the efficacy of Photofrin-photodynamic therapy.

The data presented in Figs. 2 and 3 demonstrate that the presence of prooxidants can enhance Photofrin-photosensitized lipid radical formation and cytotoxicity. Because prooxidant conditions increase free radical production, we reasoned that increasing the oxidizability of membranes would also lead to enhanced Photofrin-related free radical formation. It has been shown that cell membranes with increased levels of PUFA are more easily oxidized (8, 18). To test our hypothesis we enriched L1210 cells with docosahexaenoic acid, a highly unsaturated, 22-carbon fatty acid with 6 double bonds (22:6). Our protocol for lipid modification increases the amount of 22:6 in L1210 cells from <1% (11, 14) to approximately 30% (12–14). Membrane lipid modification produces no detectable change in Photofrin uptake. The total PUFA content increases from ≈6% to 31%. This more than doubles the average number of double bonds per fatty acid, an increase from 0.8 to 1.9 double bonds/fatty acid chain. Indeed, when 22:6-modified L1210 cells were subjected to Photofrin-photosensitization, we observed an approximate 80% increase in the amount of lipid-derived free radical formed as compared to unmodified cells (Fig. 4*A*).

The increased lipid radical formation observed in cells enriched with PUFA should correlate with increased cytotoxicity for Photofrin-photosensitization. Indeed, enhanced cell killing was observed when 22:6-enriched cells were exposed to Photofrin, Fe^{2+}, ascorbate, and light as compared to unmodified cells (Fig. 4*B*), consistent with the hypothesis that cells, with membranes that have more oxidizable substrate, are more susceptible to oxidative stress, such as presented by Photofrin-photosensitization.

In this study we have shown that: (*a*) Photofrin-photosensitization of live cells produces lipid free radicals; (*b*) iron is a key factor in the production of these free radicals, and the prooxidant combination of iron and ascorbate acts synergistically to enhance lipid radical production from Photofrin-treated live cells; (*c*) the addition of iron after light exposure of Photofrin-treated cells results in a burst of lipid radical formation and decreased cell survival; and (*d*) increasing the oxidizability of cell membranes by PUFA enrichment also results in greater lipid radical formation and decreased cell survival.

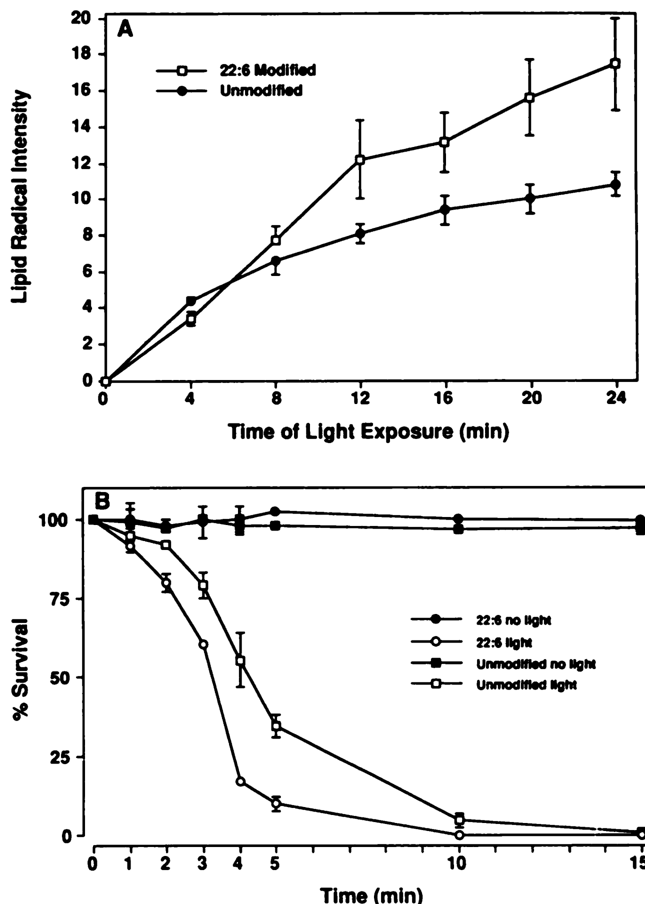

Fig. 4. Membrane lipid modification of L1210 cells. *A*, enrichment with docosahexaenoic acid (22:6), a highly unsaturated lipid, resulting in increased lipid radical formation from Photofrin-treated L1210 cells. The dark (before light exposure) POBN/lipid radical spin adduct intensity has been subtracted from each curve to show the change in lipid radical EPR signal *versus* time upon exposure to light. *Bars*, SEM of at least three replicates. The curves are significantly different at $P < 0.01$. *B*, data demonstrating that Photofrin-phototoxicity is increased in PUFA-enriched cells. Modified and unmodified L1210 cells were incubated with Photofrin, Fe^{2+}, and ascorbate and then exposed to light for various times. In the absence of light, Photofrin was not cytotoxic to either unmodified or modified cells. However, in the presence of light the PUFA-enriched cells were significantly more susceptable ($P < 0.005$ at 5 min of illumination) to the phototoxic effects of Photofrin than were modified cells.

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These *in vitro* studies suggest that the efficacy of photodynamic therapy of cancer and other diseases might be enhanced if prooxidant conditions, conditions that stimulate free radical processes, can be attained at the target locations *in vivo*. A key *in vivo* target of PDT with Photofrin is the endothelial cell of the tumor vasculature (3). These cells are inherently susceptible to free radical oxidative stress (22, 23). Thus, circulatory prooxidants, which could stimulate free radical processes, have direct contact with endothelium, thereby potentially increasing the efficacy of PDT.

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