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Effects of Endosomal Photodamage on Membrane Recycling and Endocytosis

David Kessel^{a,*}, Ann Marie Santiago^a, and Michelle Andrzejak^b

^a Department of Pharmacology, Wayne State University School of Medicine, Detroit MI 48201.

^b Department of Biology, University of Detroit Mercy, Detroit MI 48221.

Abstract

The flux of receptor-independent endocytosis can be estimated by addition of wortmannin to cell cultures. Membrane influx is unaffected but traffic out of late endosomes is impaired, resulting in a substantial enlargement of these organelles. Using the 1c1c7 murine hepatoma, we investigated the effect of endosomal photodamage on this endocytic pathway. We previously reported that photodamage catalyzed by the lysosomal photosensitizer NPe6 prevented wortmannin-induced endosomal swelling, indicating an earlier block in the process. In this study, we show that endosomal photodamage, initiated by photodamage from an asymmetrically-substituted porphine or a phthalocyanine, also prevents wortmannin-induced endosomal swelling, even when the PDT dose is insufficient to cause endosomal disruption. As the PDT dose is increased, endosomal breakage occurs, as does apoptosis and cell death. Very high PDT doses result in necrosis. We propose that photodamage to endosomes results in alterations in the endosomal structure such that influx of new material is inhibited and receptor-independent endocytosis is prevented. In an additional series of studies, we found that the swollen late endosomes induced by wortmannin are unable to retain previously accumulated fluorescent probes or photosensitizers.

INTRODUCTION

Photodynamic therapy (PDT) involves the use of photosensitizing agents that selectively localize in neoplastic tissues and their vasculature (1). Subsequent irradiation results in formation of reactive oxygen species, with cytotoxic consequences. The latter can involve apoptosis, autophagy or both (2,3), with autophagy potentially serving as either a death or a survival mechanism (3). There have been few reports linking photodamage with impairment of membrane trafficking (4,5). The latter is a process associated with endocytosis and is responsible for such processes as nutrient uptake, receptor signaling, growth, differentiation, adhesion and migration.

Pertinent information on effects of the phosphoinositol-3-kinase (PI3K) antagonist wortmannin to the interruption of endocytosis was provided by Luzio's group (6,7). Cytoplasmic vacuoles form upon exposure of mammalian cells to wortmannin. This was attributed (6) to continued endocytic plasma membrane influx into endosomes while there is a block of membrane traffic between late endosomes and lysosomes. The net effect is the appearance of swollen late endosomes.

We reported in a preliminary communication that photodamage could inhibit endocytic processes that require PI3K activity (8). Further information on is now provided by studies involving photodamage to endosomes. Agents capable of this selectivity were identified by

* Corresponding Author: dhkessel@med.wayne.edu .

Berg's group and form the basis for a therapeutic program termed 'photochemical internalization' (PCI). In PCI, irradiation of cells with photosensitized endosomes leads to endosomal disruption. This leads to an enhanced therapeutic effect when these endosomes also contain sequestered chemotherapeutic agents, e.g., the protein gelonin, which are thereby released into the cytoplasm (9-12). One report indicated that photochemical disruption of endosomes before delivery of gelonin led to a rapid release of this agent into the cytoplasm (11). This was attributed to fusion of incoming gelonin-loaded endosomes with photodamaged endosomes.

In this report, we utilized two photosensitizing agents that have successfully been employed in PCI, to further explore the role of PDT in endocytic trafficking processes. The results are consistent with the proposal that endocytosis dependent on PI3K activity can be blocked, even at level of endosomal photodamage that is insufficient to cause endosomal breakage. Additional studies on the nature of the swollen late endosomes induced by wortmannin are described.

MATERIALS AND METHODS

Drugs and chemicals

Both tetraphenyl porphine disulfonate (TPPS2a), a substituted porphine with two -SO₃H groups on adjoining phenyl rings, and the analogous disulfonated aluminum phthalocyanine (AlPcS2a) were provided by Frontier Scientific Co., Logan UT. Tissue culture media were purchased from Sigma-Aldrich, St. Louis, MO. Fluorescent probes and TDPH (trimethylamino diphenylhexatriene) were obtained from Invitrogen/Molecular Probes, Eugene OR. NPe6 (aspartyl chlorine e6) was provided by Prof Kevin M. Smith, Department of Chemistry, Louisiana State University. Other chemicals were provided by Fisher Scientific Co. (Chicago IL) and Sigma-Aldrich (St. Louis, MO).

Cells and cell culture

Maintenance of the murine hepatoma Hepa 1c1c7 cell line has been reported (13).

PDT Protocols—1c1c7 cells cultured on 22 mm glass coverslips were incubated with specified concentrations of TPPS2a or AlPc-2a for 16 h. This was followed by incubation of cells in fresh medium, usually for 1 h, to permit photosensitizers to diffuse from plasma membranes. The light source was a 600-watt quartz-halogen lamp with IR radiation attenuated by a 10-cm layer of water. Irradiation was carried out at 420 ± 10 nm (TPPS2a) or 670 ± 10 nm (AlPcS2a) as defined by interference filters.

Photosensitizer accumulation

Monolayers of 1c1c7 cells in 35 mm dishes were incubated with AlPcS2a or TPPS2a for varying intervals, then placed in fresh medium for 0-4 h. For most studies, the TPPS2a concentration = 0.2 µM and the AlPcS2a concentration = 5 µM (11,12). The intracellular concentration of photosensitizer was determined by dispersing the cells in 3 ml 10 mM Triton X-100 detergent. A sample of the medium was similarly treated. Fluorescence emission was measured at 660 (TPSP2a) or 695 nm (AlPCS2a) using broad band (350-450 nm) excitation..

Viability

1c1c7 cells were sparsely plated on 35 mm dishes. After 24 h, cultures were incubated with AlPcS2a for the times specified above in the PDT protocol. At the end of the loading period, cultures were placed in fresh medium and irradiated as specified in the text. Cultures were

then returned to the CO₂ incubator and allowed to grow until colonies could be delineated. After staining with crystal violet, colonies were counted with an Oxford Optronix 'Gelcount' colony counter. The number of colonies on control plates was approx.1000. All such experiments were carried out in triplicate.

Wortmannin-induced vacuole formation

Inhibition of PI3K-dependent processes was achieved by treatment of cultures with 20 nM wortmannin for 30 min. In studies involving PDT, cultures were treated with wortmannin for 30 min after irradiation (unless stated differently in the text), and then examined.

TDPH studies

This reagent was originally prepared with a view toward examining fluorescence polarization of membranes (14). A brief incubation (30 sec) with TDPH results in labeling of the plasma membrane only. Cultures were washed with cold medium and immediately examined by fluorescence microscopy. Alternatively, a subsequent 'chase' in fresh medium at 37°C was carried out so as to examine the migration of the probe into sub-surface regions as membrane endocytosis proceeded (15).

DEVDase assays

This follows a published procedure (2). Briefly, cells were lysed in 200 µl of buffer containing 10 mM Tris pH 7.2, 0.01% Triton X-100, 1 mM EDTA and 100 mM NaCl. Debris was removed by centrifugation at 10,000 × g for 1 min and the supernatant fluid (100 µl) mixed with an equal volume of 10 mM PIPES pH 7.5, 2 mM EDTA, 0.1% CHAPS and 10 mM DTT. To this was added zDEVD-R110 (16) (final concentration = 10 µM). The rate of increase in fluorescence, resulting from release of rhodamine-110, was measured over 30 min at room temperature using a fluorescence plate reader. Control determinations were made on untreated cells. Each assay was performed in triplicate.

Phase-contrast and fluorescence microscopy

We used phase-contrast microscopy to demonstrate the effect of different experimental conditions on cell morphology. Images were acquired with a Nikon E-600 microscope using a Photometrics Coolsnap HQ CCD camera. AlPcS2a and TPPS2a fluorescence was determined using 360–400 nm excitation and assessing 600–700 nm emission. Late endosomes/lysosomes were identified by incubating cultures with 2 µM LysoSensor Green (LSG) for 10 min at 37°C. LSG fluorescence at 500–550 nm was measured using 470 ± 20 nm excitation. Representative microscopic fields are shown; all experiments were repeated at least three times.

RESULTS

Photosensitizers

While the majority of the studies reported here were carried out with AlPcS2a, we initially employed the corresponding porphine (TPPS2a). The absorbance spectra of these agents are shown in Fig. 1. The phthalocyanine had a strong absorbance band at 673 nm; a 10 µM solution in methanol had an optical density of 2.06 using a 1 cm light path. The major absorbance for the porphine was at the Soret band (OD = 2.29 at 415 nm). The longest wavelength absorbance of the porphine shows an optical density of only 0.036 (651 nm). We used the phthalocyanine for most of the studies reported here since use of red light minimizes the light-scattering effects that are prominent at shorter wavelengths.

Accumulation of porphine and phthalocyanine sulfonates

Accumulation of AlPcS2a or TPPS2a was a linear function of the extracellular concentration over a broad concentration range. We observed a distribution ratio (intracellular vs. extracellular concentration) of 12.4 ± 1.8 for the phthalocyanine and 5.45 ± 0.71 for the porphine after 16 h incubations followed by a 1 h wash at 37°C in fresh medium. The wash removed approx. 10% of the accumulated photosensitizers. Less than 3% of the remaining intracellular pool was lost during a subsequent 1 h incubation in fresh medium at 37°C.

Wortmannin-induced vacuole formation and its inhibition

Exposure of 1c1c7 murine hepatoma cells to 20 nM wortmannin for 30 min led to the formation of the cytoplasmic vacuoles previously shown (7) to represent swollen late endosomes (Fig. 2A). Initial attempts to use flow cytometry (comparing side vs. forward scatter) to quantitate numbers of cytoplasmic vacuoles were unsuccessful, so we relied on phase-contrast microscopy to assess this phenomenon. In all reported studies, typical images are shown, representing data from triplicate experiments. To examine PDT effects, cells were incubated with 5 μ M AlPcS2a or TPPS2a for 16 hr, then resuspended in fresh medium for 1 h to remove the photosensitizer from non-endosomal loci (9-11). The cells were irradiated at 420 ± 10 nm (TPPS2a) or 670 ± 10 nm (AlPcS2a) for varying intervals at 15°C, then treated with 20 nM wortmannin for 30 min where indicated.

There was a light-dose dependent suppression of vacuole formation (Fig. 2B-D) in photosensitized cells. With a light dose of 15 mJ/cm², the number and size of the vacuoles was substantially reduced (Fig. 2B), and none were detected at 30 mJ/cm² or at higher light doses (Fig. 2C,D). Data for AlPcS2a are shown. Similar results were obtained with TPPS2a using these light doses at 420 ± 10 nm.

Effect of photodamage on endosomes

The localization of AlPcS2a is shown in Fig. 3B illustrates the endosomal pattern of fluorescence, consistent with data reported by Berg's group. When photosensitized cells were treated with a 15 mJ/cm² light dose, we observed no significant change in the labeling pattern (Fig. 3D) although this light dose was found to suppress vacuole formation (Fig. 2B). At a 30 mJ/cm² light dose, there was sufficient photodamage to cause a partial loss of photosensitizer into the cytoplasm (Fig. 3F) and an almost complete suppression of vacuole formation (Fig. 2C). At higher light doses, release of AlPcS2a into the cytoplasm was extensive and wortmannin-induced vacuole formation was absent.

Release of AlPcS2a into the cytoplasm resulted in a substantial increase in fluorescence. Panels B,D and F of Fig. 3 were acquired at 1000 msec exposure times, while panels H and J required only 500 msec.

Apoptotic effects of PDT

A 50 mJ/cm² light dose, sufficient to cause endosomal leakage of AlPcS2a (Fig. 3F), induced apoptosis in 1c1c7 cells (Fig. 4 and Table 1). Fig 4D shows the extensive apoptosis induced by a 30 mJ/cm² light dose. Arrows indicate apoptotic cells. We observed no apoptosis at 15 mJ/cm² (not shown). At very high light doses (140 and 270 mJ/cm²), apoptosis was not detected and there was a swollen cellular morphology, typical of early necrosis (Figs. 3E and G), as indicated by arrows. Effects of varying light doses on DEVDase activity (an index of apoptosis) and on viability are summarized in Table 1.

Accumulation of AIPcS2a and TPPS2a

When cells were photosensitized with NPe6 and irradiated under conditions that inhibit wortmannin-induced vacuole formation, this did not prevent AIPcS2a or TPPS2a from localizing in endosomes (Fig. 5). For this study, cells were loaded with a 40 μ M concentration of NPe6 for 60 min, then irradiated at 660 ± 10 nm (90 mJ/cm^2). This was followed by an incubation with 5 μ M concentrations of TPPS2a or AIPcS2a. The latter incubation was carried out for only 1 h, since 1c1c7 cells begin to recover from the endocytic inhibition within 3 h (5). These results indicate that the accumulation of these asymmetrically-sulfonated molecules is independent of the endocytic process that can be inhibited by NPe6-PDT (8), a procedure that targets lysosomes and endosomes for photodamage (17).

TDPH: a probe for membrane recycling

A 30 sec incubation with the fluorescent membrane probe TDPH resulted in the selective labeling of the plasma membrane (Fig. 6B). A subsequent 1 h 'chase' in fresh medium resulted in the appearance of diffuse fluorescence in the cytoplasm as membrane recycling proceeded (Fig. 6D). If cells were first loaded with AIPcS2a, then irradiated with a light dose of 30 mJ/cm^2 , the cytoplasmic TDPH labeling pattern was altered (Fig. 6F); much less diffuse cytoplasmic fluorescence was observed.

Viability data and correlations

Using a clonogenic assay, we determined the cytotoxicity of varying light doses to 1c1c7 cells loaded with AIPc-S2a and washed as described above. The data (Table 1) show that a 15 mJ/cm^2 light dose, associated with some vacuole suppression but not endosomal breakage, had almost no effect on cell viability ($\sim \text{LD}_5$). At a 30 mJ/cm^2 dose (LD_{10}), there was an almost complete vacuole suppression (Fig. 2C), accompanied by only a slight release of AIPcS2a from endosomes (Fig. 3E) and a 10% loss in viability. When the light dose reached 50 mJ/cm^2 , there was a substantial degree of apoptosis (Table 1), substantial endosomal breakage resulting in release of the photosensitizer into the cytoplasm (Fig. 3H) and a 50% loss of viability. At a still higher light doses (90 mJ/cm^2 , LD_{90}), the degree of cellular photodamage resulted in a marked decrease in apoptosis with the cells showing a swollen morphology consistent with necrosis.

Effects of wortmannin on endosomal integrity

Swollen endosomes that form when 1c1c7 cells are exposed to wortmannin were unable to retain endosomal probes or photosensitizers. In one study, 1c1c7 cells were first loaded with 5 μ M AIPcS2a during a 16 h incubation, then resuspended in fresh medium for 1 h with 20 nM wortmannin added during the last 30 min. The resulting labeling pattern is shown in Fig. 7. Swollen late endosomes can clearly be seen in the phase-contrast image (Fig 7A). The fluorescence image reveals that these contain no AIPcS2a with all fluorescence appearing in the cytoplasm (Fig. 7B). When photosensitized cells were given a 30 mJ/cm^2 light dose before addition of wortmannin, we observed no loss of the endosomal fluorescence-labeling pattern (Fig 7C).

In another study, we first labeled endosomes/lysosomes with LSG during a 10 min incubation in medium containing a 5 μ M concentration of this agent. A subsequent incubation in fresh media containing 20 nM wortmannin for 30 min also resulted in the migration of label into the cytoplasm.

DISCUSSION

To assess the 'flux' of autophagy, inhibitors of lysosomal proteases are often used to interrupt further processing of autophagosomes (18). We propose that wortmannin can be similarly used to probe receptor-independent endocytic processes. A block in membrane trafficking by this PI-3K antagonist leads to formation of swollen late endosomes, if the endocytic pathway is functional. In the present study, we show that even sub-lethal photodamage to endosomes can markedly reduce swollen late endosome formation induced by wortmannin. (Figs. 2 and 3).

We previously demonstrated that photodamage catalyzed by the chlorin NPe6 suppressed membrane trafficking that required PI3K activity (5). We observed that NPe6-induced photodamage did not prevent AlPcS2a from localizing in endosomes (Fig. 5). This result indicates that endosomal accumulation of AlPcS2a can occur even where further steps in the endocytic membrane-recycling pathway are impaired or blocked.

Additional evidence that endosomal photodamage can affect membrane recycling processes was provided by studies involving the plasma membrane probe TDPH (Fig. 6). This probe normally reaches the interior of the cytoplasm within 30 min, but this migration was reduced by prior endosomal photodamage.

When the degree of photodamage was sufficient to disrupt endosomes, the resulting release of endosomal contents forms the basis for the PCI process described by Berg's group (9-11). We have now characterized a series of dose-dependent effects evoked by PDT that relate to both membrane trafficking and cell death mechanisms. Low-dose endosomal photodamage can lead to interference with PI3K-dependent membrane trafficking processes without disrupting endosomes; as the PDT dose is increased, endosomes are disrupted. This is accompanied by release of AlPcS2a into the cytoplasm, along with appearance of apoptotic cells (Fig. 4, Table 1). When the affected endosomes have sequestered chemotherapeutic agents, this level of photodamage will lead to release of such agents, with additional cytotoxic consequences as indicated by Berg's reports (9-11). A very high PDT dose can result in photodamage to elements of the apoptotic process, and appearance of the swollen cells that are characteristic of necrosis (Fig. 4). This effect has been previously described before in the context of PDT (19).

Exposure of 1c1c7 cells containing endosomal AlPcS2a to wortmannin led to the quantitative migration of fluorescence into the cytoplasm (Fig. 7) as swollen late endosomes began to appear. A prior low level of endosomal photodamage prevented this release of AlPcS2a (Fig. 7C). This provides further evidence that effects of wortmannin resulting in swollen late endosome formation and abrogated by endosomal photodamage.

We conclude that a low level of endosomal photodamage does not result in loss of endosomal integrity, but does affect the ability of the affected endosomes to participate in membrane trafficking processes. In the presence of wortmannin, swollen late endosomes are produced, but not after endosomal photodamage insufficient to cause disruption. Receptor-mediated endocytosis, e.g., EGF transfer from the cell surface to the nucleus mediated by EGFR, is not affected by wortmannin (20). This may explain studies indicating that PDT does not affect EGF trafficking (21). Since this is among the first reports on a PDT-induced inhibition of membrane recycling, it is not yet clear what effect this might have on the overall effect of photodamage or whether other anti-tumor modalities might also evoke this response.

Acknowledgments

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Figure 1.
Absorbance spectra of 10 μ M AlPcS2a (solid line) and TPPS2a (dotted line) in ethanol.

Figure 2.

Production of swollen late endosomes 30 min after treatment of 1c1c7 cells with 20 nM wortmannin. A, no additional treatment; B-E, after irradiation of cells (670 ± 10 nm) previously loaded with 5 μ M AIPCS2a as described in the text: B = 15 mJ/cm²; C = 30 mJ/cm², D = 50 mJ/cm², E = 140 mJ/cm² of light at 670 ± 10 nm. White bar in panel A = 20 μ m.

Figure 3. Phase-contrast and fluorescent images of cells loaded with 5 μM AlPcS2a as described in the text then irradiated at 670 ± 10 nm with varying light doses (mJ/cm^2): A,B = 0; C,D = 15; E,F = 30; G,H = 55; I,J = 140. White bar in panel A = 20 μm .

Figure 4.

Phase contrast (left) and fluorescence (right) images of 1c1c7 cells loaded with 5 μ M ALPcS2a as described in the text, then irradiated (670 ± 10 nm) with 0 (A), 55 (B), 140 (C) or 270 (D) mJ/cm^2 . Cells were subsequently incubated for 60 min at 37°C with HO342 added for the last 10 min of these incubations. White bar in panel A = 50 μm . Arrows in panels C and D indicate apoptotic cells; in panel G, swollen pre-necrotic cells.

Figure 5.

Localization of TPPS2a and AlPcS2a. Left panels: phase contrast images; right panels, fluorescence. A,B = TPPS2a after a 1 hr incubation. C,D = TPPS2a uptake directly following photodamage induced by NPe6 (LD_{30}). E,F = AlPcS2a uptake after 1 hr. G,H = AlPcS2a uptake following NPe6-induced photodamage. White bar in panel A = 20 μ m.

Figure 6.

Labeling of 1c1c7 cells by TDPH. Left panels, phase contrast; right panels, fluorescence. A = TDPH 30 sec incubation B = TDPH 30 sec incubation a 30 min incubation in fresh medium at 37°; C. C, Similar to B using cells loaded with 5 μ M AIP2a and irradiated (30 mJ/cm^2). White bar in panel A = 20 μ m.

Figure 7.

Cells incubated with 5 μM AlPcS2a for 16 hr, then washed for 1 h with 20 nM wortmannin added during the final 30 min. A = phase contrast; B = fluorescence. Panel C shows the effect of a 30 mJ/cm^2 light dose before addition of wortmannin. White bar in panel A = 20 μm .

Figure 8.

Release of LSG by wortmannin. Cells were incubated with 1 μ M LSG for 10 min, then placed in fresh medium for 30 min at 37°C (A), or in medium containing 20 nM wortmannin for 30 min (B). White bar in panel A = 20 μ m.

Table 1

Effects of light on DEVDase activity and viability of 1c1c7 cells

light dose (mJ/cm ²)	DEVDase (nmol/min/mg protein)	viability
0	0	100
15	0.38 ± 0.04	95 ± 3
30	0.84 ± 0.62	89 ± 4
50	6.4 ± 0.58	63 ± 3
90	4.9 ± 0.3	9.2 ± 2
135	1.1 ± 0.02	< 2
180	0.82 ± 0.02	< 1