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Release of cytochrome *c* and activation of pro-caspase-9 following lysosomal photodamage involves bid cleavage

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Abstract

Photodynamic therapy (PDT) protocols employing lysosomal sensitizers induce apoptosis via a mechanism that causes cytochrome *c* release prior to loss of mitochondrial membrane potential ($\Delta\Psi_m$). The current study was designed to determine how lysosomal photodamage initiates mitochondrial-mediated apoptosis in murine hepatoma 1c1c7 cells. Fluorescence microscopy demonstrated that the photosensitizer N-aspartyl chlorin e6 (NPe6) localized to the lysosomes. Irradiation of cultures preloaded with NPe6 induced the rapid destruction of lysosomes, and subsequent cleavage/activation of Bid, pro-caspases-9 and -3. Pro-caspase-8 was not activated. Release of cytochrome *c* occurred at about the time of Bid cleavage and preceded the loss of $\Delta\Psi_m$. Extracts of purified lysosomes catalyzed the *in vitro* cleavage of cytosolic Bid, but not pro-caspase-3 activation. Pharmacological inhibition of cathepsin B, L and D activities did not suppress Bid cleavage or pro-caspases-9 and -3 activation. These studies demonstrate that photodamaged lysosomes trigger the mitochondrial apoptotic pathway by releasing proteases that activate Bid.

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Keywords: apoptosis; Bid; lysosomes; NPe6; photodynamic therapy (PDT)

Abbreviations: AFC, 7-amino-4-(trifluoromethyl)coumarin; AMC, 7-amino-4-methylcoumarin; AO, acridine orange; CPO, 9-capronyloxy-tetrakis(methoxyethyl) porphycene; Ac-IETD-AFC, N-acetyl-Ile-Glu-Thr-Asp-aminofluorocoumarin; Ac-DEVD-AMC, N-acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin; HPPH, hexyl pheophorbide; HO342, Hoechst dye HO33342; LTB, LysoTracker Blue; LuTex, lutetium texaphyrin; NPe6, N-aspartyl chlorin e6; Pc 4, phthalocyanine; PDT, photodynamic therapy; $\Delta\Psi_m$, mitochondrial membrane potential; tBid, truncated Bid; TMRM, tetramethylrhodamine methyl ester; Z-FA-FMK, Z-Phe-Ala-fluoromethyl ketone

Introduction

PDT is a procedure that employs light to activate photosensitizers and generate singlet oxygen. It is used to eradicate tumors, and in the treatment of atherosclerotic plaque and macular degeneration.[1] It was originally assumed that PDT caused necrotic cell death. However, in 1991 Agarwal *et al*.[2] showed that apoptosis could also occur. Subsequent studies demonstrated PDT-mediated apoptotic/necrotic outcomes in various cell types,[1,3] with the mode of cell death being influenced by cell type, identity and concentration of photosensitizer, and light doses used in the PDT protocol.[3]

A variety of agents function as photosensitizers in PDT protocols. However, the initial intracellular target of photosensitizers is agent specific.[1,3,4] For example, the photosensitizers LuTex and NPe6 cause an almost immediate disruption of the lysosomes upon irradiation.[1,3–6] In contrast, irradiation of cells preloaded with the photsensitizers CPO, HPPH and Pc 4 has no effect on lysosomes. Instead, one sees a rapid loss of $\Delta\Psi_m$ and release of cytochrome *c*.[1,3,7–9] These effects most likely reflect: (a) the sites of sensitizer accumulation[6,10,11] because the reactive oxygen species formed upon irradiation have a limited ability to migrate from the site(s) of formation;[12] and (b) the unique ability of some photosensitizers to photo oxidize and cleave Bcl-2 upon excitation.[13,14]

Irradiation of cells preloaded with lysosomal photosensitizers eventually causes the release of cytochrome *c* and the activation of pro-caspase-3.[5,8] The mechanisms by which lysosomal sensitizers cause these effects are not known. However, one can envision several possibilities. For example, several investigators have reported that extracts of purified lysosomes can directly activate pro-caspases *in vitro*.[15–18] Alternatively, released lysosomal proteases may directly attack the mitochondria causing damage sufficient to facilitate the release of cytochrome *c*. Cytosolic cytochrome *c* could then activate pro-caspase-3 via Apaf-1/caspase-9. Released lysosomal proteases may also activate pro-apoptotic cytosolic proteins capable of triggering cytochrome *c* release. Indeed, Stoka *et al*.[19] recently demonstrated that lysosomes contain a proteolytic activity capable of the *in vitro* conversion of Bid to tBid, a pro-apoptotic member of the *Bcl-2* supergene family.[20,21] tBid, like the pro-apoptotic protein Bax, can trigger the release of cytochrome *c* from isolated mitochondria.[22–24] It is currently thought that tBid induces the intra-membranous

oligomerization of the pro-apoptotic protein Bak into a pore that facilitates the release of cytochrome *c*.[25–27] The cytochrome *c* release catalyzed by tBid occurs in the absence of a loss of $\Delta\Psi_m$, and cannot be suppressed by cyclosporin A or bongkrekic acid, inhibitors of mitochondrial permeability transition.[22–24]

We previously reported that irradiation of murine hepatoma 1c1c7 cultures preloaded with NPe6 caused lysosomal disruption, dispersion of lysosomal proteases throughout the cytosol, and apoptosis.[5] In the current study we determined if Bid activation occurs in this PDT protocol. Our studies demonstrate that Bid cleavage does occur and precedes/accompanies the initiation of cytochrome *c* release and activation of the Apaf-1/pro-caspase-9 pathway. Cytochrome *c* release occurred prior to the loss of $\Delta\Psi_m$. Furthermore, we demonstrate that extracts of purified lysosomes can convert cytosolic Bid to tBid, but can not directly activate pro-caspase-3.

Results

Localization of NPe6 to lysosomes

1c1c7 cultures preloaded with NPe6 exhibited punctate, perinuclear fluorescence when excited (Figure 1A). In order to determine if the staining pattern represented lysosomes we attempted to colocalize NPe6 staining with LTB fluorescence. LTB is an acidophilic dye commonly used to localize lysosomes and endosomes. LTB also exhibited a punctate, perinuclear staining pattern in 1c1c7 cultures (Figure 1B). The merged stained images revealed that NPe6 colocalized to a subset of the LTB-stained structures (Figure 1C).

Cell killing and activation of pro-caspase-3 by NPe6 in PDT protocols

Exposure to either 22 or 66 μM NPe6 for 1 h, in the absence of light, was not cytotoxic to 1c1c7 cultures (time 0 in Figure 2A). Similarly, in the absence of photosensitizer, cell viability was unaffected by 140 s of irradiation (210 mJ/cm^2, Figure 2A). However, irradiation of cultures preloaded with either 22 or 66 μM NPe6 resulted in light-dose-dependent, and sensitizer-concentration-dependent cell killing (Figure 2A). The LD_{50s} for 22 and 66 μM NPe6 were $\sim$165 mJ/cm^2 (110 s of irradiation) and 37.5 mJ/cm^2 (25 s of irradiation), respectively.

Reductions in viability, as scored in colony formation assays, were also paralleled by the activation of pro-caspase-3 (Figure 2B,C). Neither irradiation alone, nor NPe6 alone, activated pro-caspase-3. However, when used in combination, pro-caspase-3 activation occurred in a light-dose-dependent, and sensitizer-concentration-dependent fashion (Figure 2B,C). Caspase-3 activities were elevated slightly within 2 h of irradiation of sensitized cultures, and generally peaked within an additional 3–5 h (Figure 2B,C). Maximum increases of $\sim$200-fold were measured in cultures treated with 66 μM NPe6 and $\geqslant$90 mJ/cm^2 ($\geqslant$60 s of irradiation). Light microscopy revealed that most cells in cultures sensitized with 66 μM NPe6 were undergoing apoptosis within 6 h of irradiation with 135 mJ/cm^2 (90 s of irradiation). In subsequent PDT studies, unless stated otherwise, cultures were treated with 66 μM NPe6 and 135 mJ/cm^2.

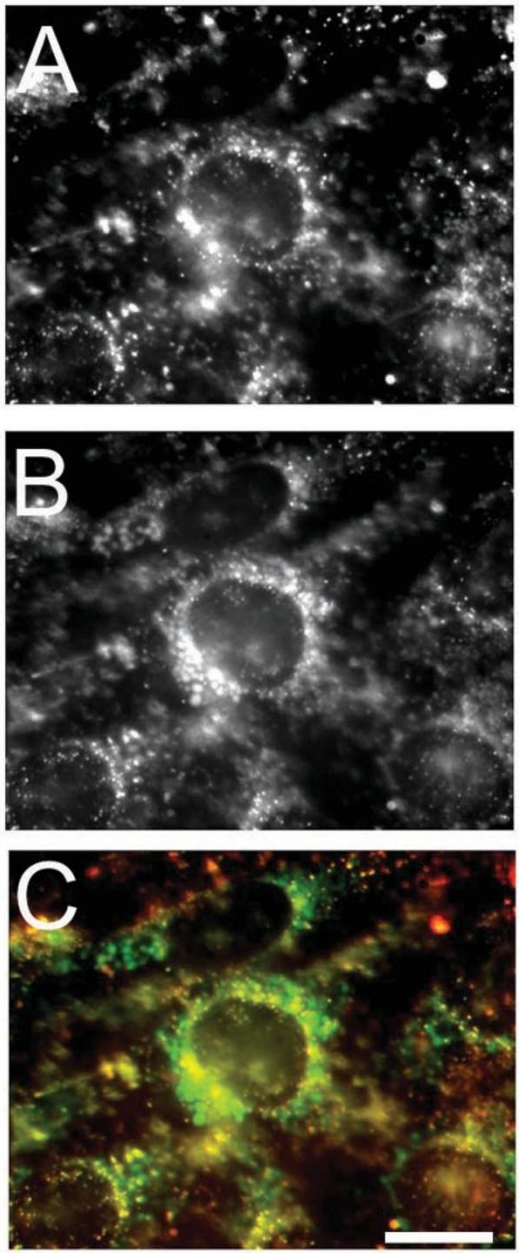

Figure 1 Localization of NPe6 in 1c1c7 cells. Cells grown on coverslips were preloaded with 66 μM NPe6 for 30 min prior to being washed, refed and loaded with LysoTracker Blue (LTB). After 10 min the cultures were washed with PBS and analyzed by fluorescence microscopy. Panels represent fluorescence of: (**A**) NPe6, (**B**) LTB, (**C**) merged image of NPe6+LTB. Bar represents 20 microns

PDT induction of apoptosis

AO stained lysosomes in non-treated, control cultures exhibited a punctate pattern (Figure 3). Irradiation of cultures preloaded with NPe6 almost completely abolished AO staining. This effect was observed within 1 h of irradiation.

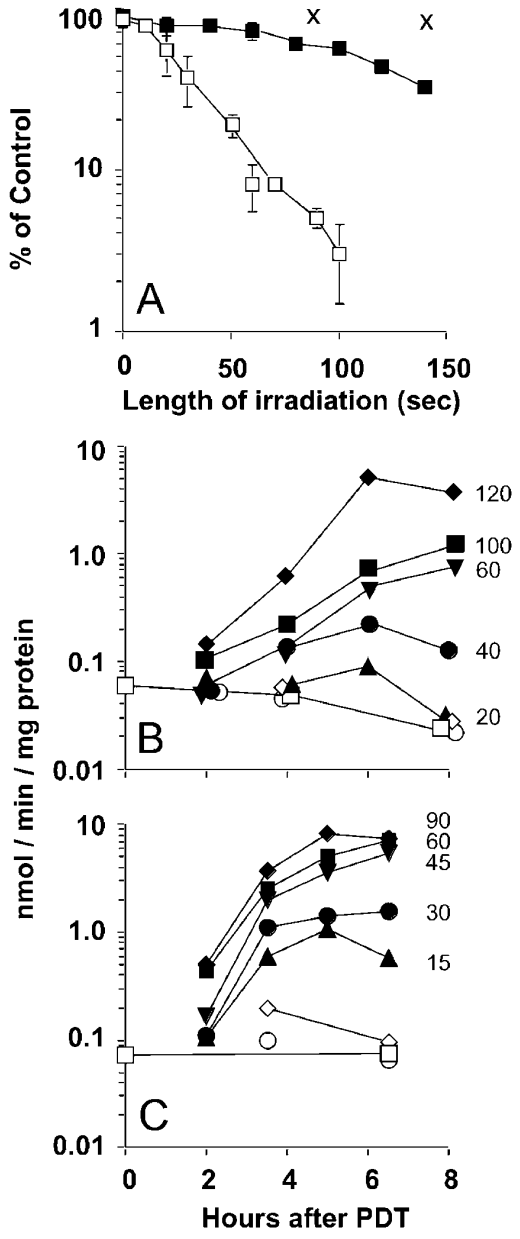

Figure 2 Light dose and NPe6-concentration-dependent killing and activation of caspase-3 in PDT protocols. (**A**) 1c1c7 cells were plated at densities of 400–800 cells/60 mm dish. Approximately 16 h later cultures were loaded with 22 μM (■) or 66 μM (□) NPe6 for 45 min prior to being washed, refed and irradiated for the indicated period of time. Other cultures were only irradiated (X). Colonies were counted 7–9 days after irradiation. Data represent means $\pm$ S.D. of 3–4 plates. (**B**) Subconfluent, 2 day-old cultures were loaded with 22 μM NPe6 for 45 min prior to being washed, refed and irradiated for 20–120 s (time is indicated next to solid symbol). Cultures were harvested at various times after irradiation for assay of caspase-3 activities. (**C**) Same as **B** except that cultures were preloaded with 66 μM NPe6. Open symbols in **B** and **C** represent: no treatment (□), light alone (◇), and NPe6 alone (○). Data in panels B and C represent means $\pm$ S.D. of triplicate assays of a single culture. One s of irradiation=1.5 mJ/cm^2. Similar results were obtained in a second independent experiment

In contrast, AO staining was unaffected in parallel cultures that were irradiated in the absence of NPe6, or treated solely with NPe6 (data not presented).

TMRM is a fluorescent probe used to monitor $\Delta\Psi_m$. Non-treated, control cells stained with TMRM exhibited a perinuclear punctate pattern (Figure 3). No dramatic changes in either the pattern or the intensity of TMRM staining occurred within 4 h of irradiation of NPe6-sensitized cultures (Figure 3). However, TMRM staining intensity decreased dramatically within 5 h of PDT (Figure 3), and was almost uniformly absent within 6 h of PDT (data not presented).

Analyses of morphology and nuclear condensation (Figure 3) indicated that some of the cells in irradiated 1c1c7 cultures preloaded with NPe6 were undergoing apoptosis within 4 h of irradiation. By 6 h a large percentage of the cells had detached, all of which where apoptotic. Of the remaining attached cells, most exhibited nuclear condensation.

The conversion of cytosolic Bid to tBid occurred within 3 h of PDT (Figure 4A). Analyses employing ECL exposure times different than those used to generate the data depicted in Figure 4 clearly demonstrated that the appearance of tBid was accompanied by the disappearance of Bid (data not presented). Cytosolic cytochrome *c* contents were elevated within 3 h of PDT and continued to increase with time (Figure 4A). The appearance of cytochrome *c* was paralleled by the cleavage/activation of pro-caspase-9 (Figure 4A). Significant increases in caspase-3 activity occurred only after the activation and appearance of caspase-9 (Figure 4B). Specifically, in the study reported in Figure 4, caspase-3 activity was unaffected for the first 2 h after PDT. However, within 3 and 4 h of PDT caspase-3 activities were $\sim$7 and 20-fold higher than the activities measured in non-treated cultures, respectively. Thereafter, caspase-3 activity continued to increase. Neither irradiation alone, nor exposure to only NPe6, activated pro-caspase-3 (Figure 4B).

Peng *et al*.[28] demonstrated that PDT sensitizers sequestered in organelles can be released into the cytoplasm following irradiation under some circumstances, and undergo subsequent photoactivation in the cytoplasm if sufficient light is present. Furthermore, recent studies demonstrate that several mitochondrial sensitizers, which are normally photoactivated in the cytoplasm of the cell, cause photocleavage of Bcl-2 in PDT protocols.[13,14] Cultures were irradiated for 90 s in the studies presented in Figure 4 (LD_{98} conditions). At issue is whether the Bid cleavage seen in Figure 4 reflects the actions of a released lysosomal protease, or photocleavage mediated by NPe6 that is released from the lysosome and subsequently activated in the cytosol. To address this issue we repeated the studies presented in Figure 4, but shortened the irradiation time to 45 s (LD_{75} conditions). Irradiation for 45 s also resulted in Bid cleavage, and pro-caspases-9 and -3 activation (Figure 5A). tBid and processed caspase-9 were clearly present within 4 h of irradiation. Caspase-3 activities were also significantly elevated ($\sim$70-fold) within 4 h of irradiation. By employing shorter ECL exposure times it was possible to demonstrate a time-dependent loss of Bid

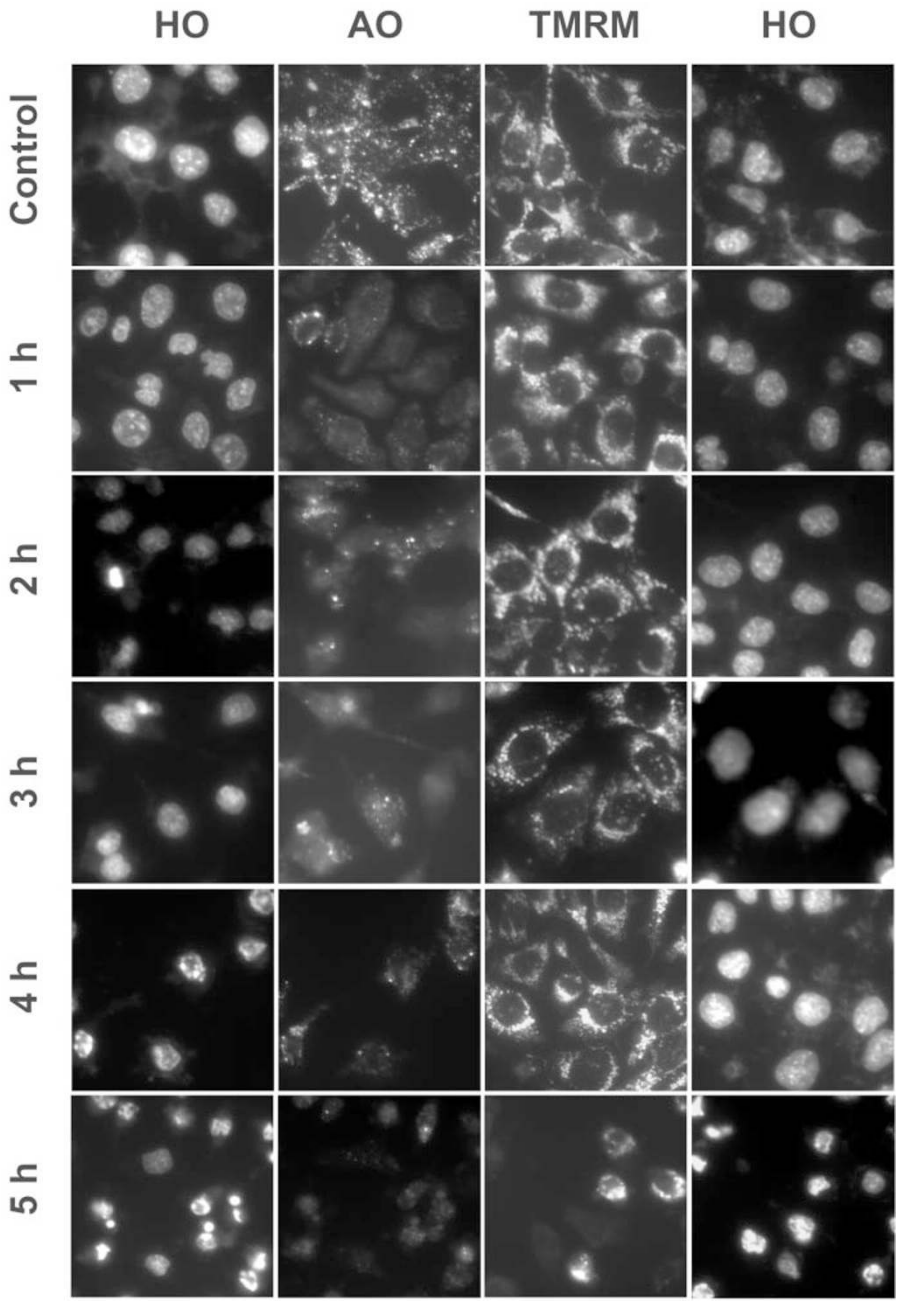

Figure 3 Effects of PDT with NPe6 on lysosomal integrity and mitochondrial membrane potential. Cells grown on coverslips were preloaded with 66 μM NPe6 for 30 min prior to being washed, refed and irradiated (135 mJ/cm^2). PDT-treated cultures were co-stained at the indicated times after irradiation with either HO+AO (first and second columns), or TMRM+HO (third and fourth columns) to visualize nuclei+lysosomes and $\Delta\Psi_m$+nuclei, respectively. Non-treated control cultures were treated and processed similarly. Similar results were obtained in a second independent experiment

in irradiated, NPe6-sensitized 1c1c7 cultures (Figure 5A). The Bid signal was reduced ~25% and ~85% within 3 and 6 h of irradiation, respectively.

Incubation of cytosol isolated from normal cultures with NPe6 for 6 h, in the absence of light, did not cause Bid cleavage and the formation of tBid (Figure 5B). Similarly, irradiation of cytosol for 90 s, in the absence or presence of NPe6, did not stimulate Bid loss and the formation of tBid over a 6 h time period (Figure 5B). This resistance of Bid to 90 s of irradiation *in vitro*, in the presence of NPe6, is in marked contrast to the dramatic loss of Bid that occurred *in vivo* following 45 s of irradiation of NPe6 sensitized cultures (compare 6 h Bid signal in Figure 5A with Bid signal in Figure 5B). Hence, the tBid formed in our PDT protocols was not the consequence of NPe6-induced Bid photocleavage.

In vitro cleavage of Bid and activation of pro-caspase-3 by lysosomal extracts

1c1c7 cells were broken by gentle homogenization to yield cytosol and organelle fractions. Purified cytosol was devoid of lysosomal proteins (cathepsin D and β-hexosaminidase) and the mitochondrial marker cytochrome *c* (Figure 6A), but contained Bid (Figure 6B). Density gradient centrifugation of the organelle fraction yielded a lysosome preparation that contained β-hexosaminidase and mature single chain and large chain forms of cathepsin D, but no cytochrome *c* (Figure 6A), or Bid (Figure 6B). Incubation of the cytosolic fraction with lysosomal extract resulted in time-dependent Bid cleavage and the formation of tBid (Figure 6B). No cleavage occurred in

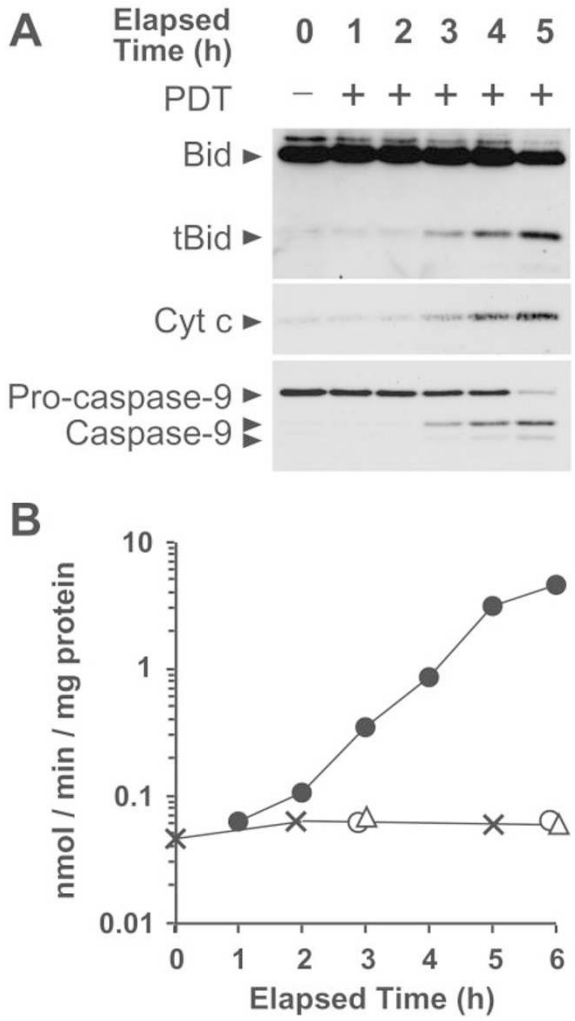

Figure 4 Kinetics of Bid cleavage, cytochrome *c* release, and activation of pro-caspase-9 and pro-caspase-3 following high light dose irradiation of NPe6-sensitized 1c1c7 cells. Two-day-old cultures were preloaded with 66 μM NPe6 for 45 min prior to being washed, refed and irradiated (135 mJ/cm^2). Cultures were harvested at various times after irradiation for analyses of: (**A**), Bid cleavage, cytosolic cytochrome *c*, and pro-caspase-9 cleavage; and (**B**), caspase-3 activity. Parallel cultures were either not treated, or only irradiated, or only sensitized with NPe6. Symbols in **B** are means $\pm$ S.D. of triplicate assays of a single culture and represent: no treatment (X); irradiation alone (△); NPe6 alone (○); and NPe6+irradiation (●). Western blot analyses used 20, 10, and 20 μg of protein per lane for Bid, cytosolic cytochrome *c* and caspase-9 analyses, respectively. Similar data were obtained in a second independent study

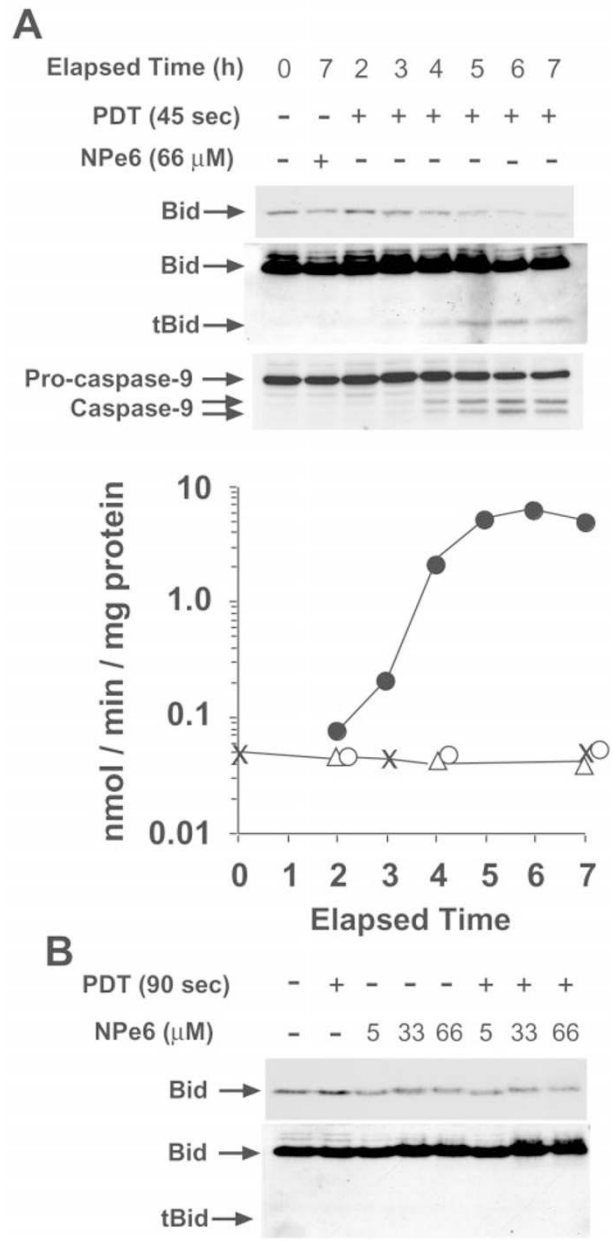

Figure 5 Kinetics of Bid cleavage and pro-caspases-9 and -3 activation following low light dose irradiation of NPe6-sensitized 1c1c7 cells. (**A**) Two day-old cultures were preloaded with 66 μM NPe6 for 45 min prior to being washed, refed and irradiated (67.5 mJ/cm^2). Cultures were harvested at various times after irradiation for analyses of Bid and pro-caspase-9 cleavage and caspase-3 activity. Parallel cultures were either not treated, or only irradiated, or only sensitized with NPe6. Symbols are means $\pm$ S.D. of triplicate assays of a single culture and represent: no treatment (X); irradiation alone (△); NPe6 alone (○); and NPe6+irradiation (●). (**B**) Cytosolic extracts were incubated in the presence of varied concentrations of NPe6 for 6 h, or irradiated for 90 s (135 mJ/cm^2), and then incubated for 6 h prior to being used for analyses of Bid and tBid. Parallel cultures received varying concentrations of NPe6 prior to being irradiated and incubated an additional 6 h. Western blot analyses used 20 μg of protein per lane. ECL exposure times were varied to facilitate analyses of Bid loss and tBid appearance

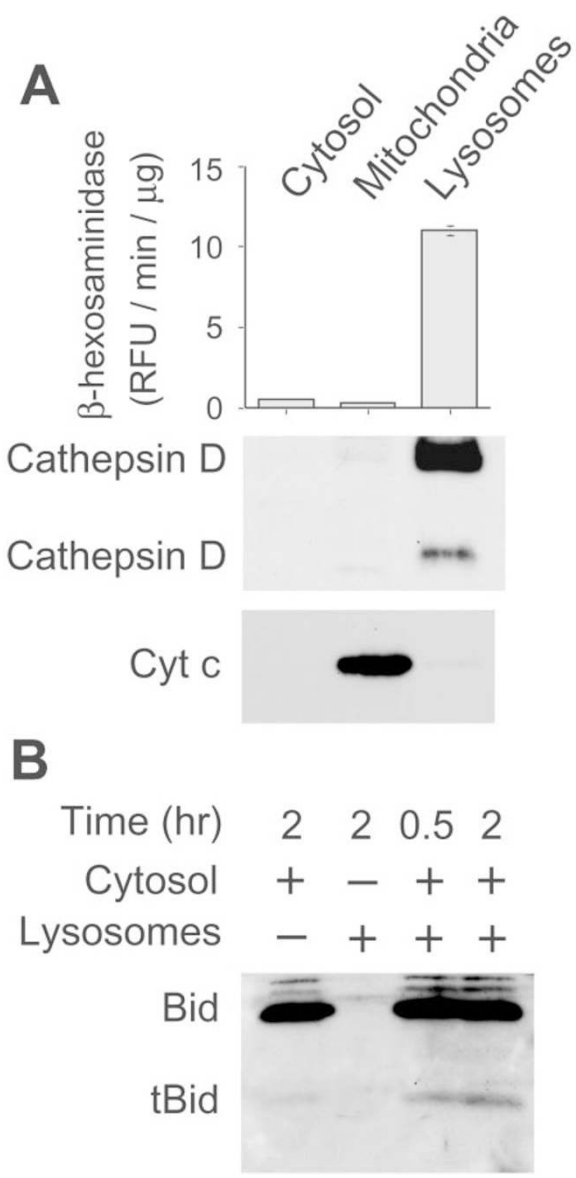

Figure 6 *In vitro* cleavage of Bid by lysosomal extracts. (**A**) Cytosol and extracts from purified lysosomes and mitochondria were assayed for β-hexosaminidase activity, and the presence of cathepsin D and cytochrome *c*. Western blot analyses used 20 μg of protein per lane. (**B**) Cytosol (20 μg) and lysosomal extract (2.5 μg) were mixed and incubated for either 30 or 120 min prior to being used in Western blot analyses. Cytosol and lysosomal extracts were incubated in parallel for 120 min prior to analyses. Western blot analyses in **B** used 20, 2.5 and 22.5 μg of cytosolic, lysosomal, or cytosolic+lysosomal protein, respectively. Similar results were obtained in a second experiment employing a different preparation of lysosomes and cytosol

the absence of lysosomal extract. Hence, lysosomes isolated from 1c1c7 cells contain a protease(s) capable of either directly cleaving Bid to tBid, or activating a cytosolic protease capable of the cleavage.

Incubation of cytosol with varied amounts of lysosomal extract over the pH range 5.5–7.2, for 5–30 min, at 37°C did not activate cytosolic pro-caspase-3 (data not presented). However, cytosolic pro-caspase-3 could be activated by the addition of caspase-9. Addition of lysosomal extracts to cytosols containing active caspase-3 had no effects on pre-existing caspase-3 activity (data not presented). Hence, the inability of lysosomal extract to activate pro-caspase-3 did not reflect the presence of a suppressive activity.

Role of cathepsin D and cysteine cathepsins in Bid cleavage and pro-caspase-3 activation

Lysosomes/endosomes contain numerous proteolytic enzymes including the aspartic protease cathepsin D, and several cysteine proteases (e.g., cathepsins B and L).[29] Several studies implicate a role for these lysosomal proteases in the activation of pro-caspases and the initiation and excutionary phases of apoptosis.[30–34] Z-FA-FMK is a potent, irreversible inhibitor of cathepsins B and L.[35] We have determined that intracellular cathepsin B and L activities are suppressed totally within 30 min of exposure of 1c1c7 cultures to 1 μM Z-FA-FMK, and remain suppressed for at least an additional 24 h.[36] Co-incubation of 1c1c7 cultures with 1 μM Z-FA-FMK at the time of NPe6 loading altered neither the kinetics or magnitude of caspase-3 activation, nor the cleavages of Bid or pro-caspase-9 following irradiation (Figure 7A,E).

Pepstatin A is a potent inhibitor of cathepsin D. It is commonly used in the 50–150 U/ml range, and added to cultures 12–24 h prior to initiation of treatment in order to suppress cathepsin D activity.[33,34] Pretreatment of 1c1c7 cultures with 100 U/ml of pepstatin A for 18 h prior to irradiation of NPe6-sensitized cultures did not suppress pro-caspase-3 or pro-caspase-9 activation (Figure 7C,E) or Bid loss and formation of tBid (Figure 7E).

Activation of pro-caspase-8 in lysosomal PDT protocols

The fluorogenic tetrapeptide substrate Z-IETD-AFC is cleaved by caspase-8 and granzyme B. Either protease can cleave Bid to tBid.[37,38] However, we anticipated that 1c1c7 cells would contain only pro-caspase-8 because of the restricted expression of granzyme-B to immune cells. Irradiation of NPe6-sensitized 1c1c7 cultures elevated caspase-8 specific activities ~5-fold over a 6–7 h period (Figure 7B,D). In contrast, caspase-3 specific activities were elevated ~200-fold during the same period (Figure 7A,C). Furthermore, pro-caspase-3 activation preceded pro-caspase-8 activation by at least 2 h (compare Figure 7A with B, and C with D). Hence, caspase-8 does not contribute to Bid cleavage in our PDT protocol.

Discussion

We recently reported that PDT protocols employing lysosomal photosensitizers cause an immediate disruption of the lysosomes, and a subsequent, but delayed release of cytochrome *c* and activation of pro-caspase-3.[5] How lysosome disruption led to cytochrome *c* release and pro-caspase-3 activation was unclear. Several studies circumstantially suggest that lysosomes contain one or more

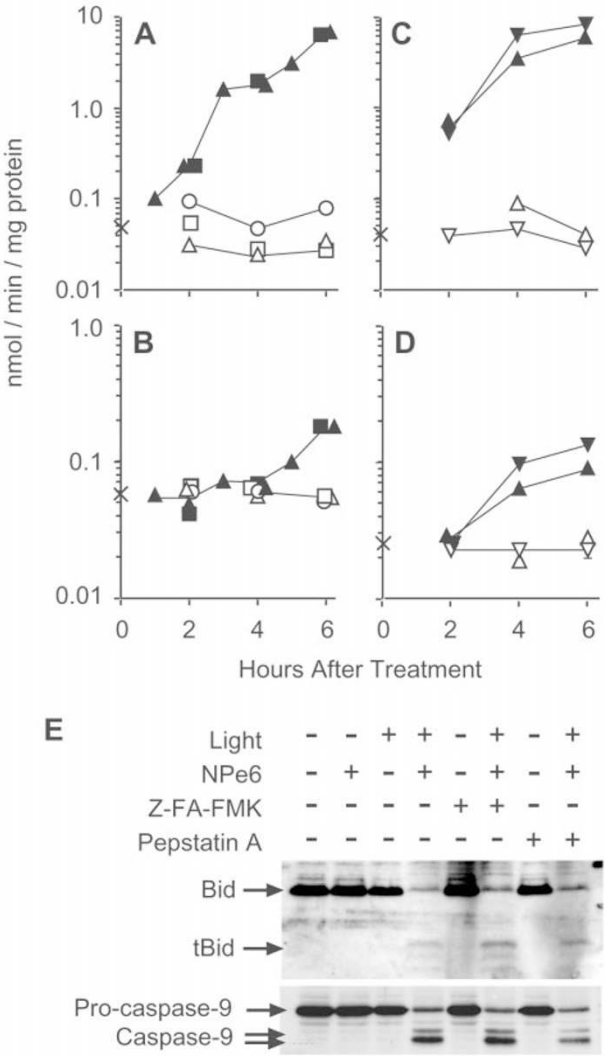

Figure 7 Effects of Z-FA-FMK and pepstatin A on NPe6/PDT-induced apoptosis. Two-day-old 1c1c7 cultures were co-treated with either 1 μM Z-FA-FMK at the time of NPe6 loading (**A,B,E**), or treated with pepstatin A (100 units/ml) 18 h prior to NPe6 loading (**C,D,E**). Cultures were loaded with NPe6 for 45 min prior to being washed, refed and irradiated (180 mJ/cm^2). Cultures were subsequently harvested 1 – 6 h after irradiation for analyses of caspase-3 (**A,C**) and caspase-8 (**B,D**) activities. Other cultures were harvested 5 h after irradiation for Western blot analyses of Bid (**E**). Data in **A – D** represent means $\pm$ S.D. of triplicate assays of a single culture. Symbols are: no treatment (X), light (○), NPe6 (△), Z-FA-FMK (□), pepstatin A (▽), NPe6+light (▲), NPe6+ light+Z-FA-FMK (■), and NPe6+light+pepstatin A (▼). Western blot analyses used 40 μg of protein per lane. Similar results were obtained in a second independent experiment

proteases capable of directly activating pro-caspase-3.[13–16] The existence of such a protease would provide a plausible explanation for how lysosome disruption causes pro-caspase-3 activation. Furthermore, Marzo *et al*.[39] demonstrated that isolated mitochondria undergo a loss of $\Delta\Psi_m$ and release cytochrome *c* upon incubation with purified caspases. However, our observations are not consistent with a model entailing direct lysosomal protease activation of pro-caspase-3, and subsequent caspase-3 damage of mitochondria. Specifically, in our NPe6 PDT protocol pro-caspase-3 activation did not occur prior to cytochrome *c* release and pro-caspase-9 cleavage (Figure 4). Furthermore, *in vitro* incubation of cytosol with a lysosomal extract capable of activating Bid did not activate pro-caspase-3.

Bid is a pro-apoptotic member of the Bcl-2 supergene family that is activated by limited proteolysis.[20,21,37,38] The cleavage product tBid can stimulate the release of cytochrome *c* from mitochondria.[21,22] The proteolytic activation of Bid can be catalyzed by caspase-8, granzyme B, and an unidentified lysosomal protease.[19,37,38] Caspase-8 activity was elevated following the irradiation of NPe6-sensitized 1c1c7 cultures. However, the elevations (5 – 7-fold above basal activities) were diminutive relative to caspase-3 increases ($\sim$200-fold), and occurred after caspase-3 activation and Bid cleavage (Figure 7). Hence, it is unlikely that caspase-8 contributed to Bid cleavage in our system. Instead, a lysosomal protease is most likely responsible for Bid cleavage in our PDT model. This conclusion is based upon two findings. First, inculbation of cytosol with extracts of purified 1c1c7 lysosomes caused Bid cleavage and the formation of tBid (Figure 6). Second, PDT protocols with NPe6 caused a targeted disruption of the lysosomes. Manipulation of the PDT protocol so as to suppress lysosome disruption (e.g., exclusion of NPe6 or no irradiation) inhibited Bid cleavage. At face value, the only data casting doubt on lysosomal protease-mediated Bid cleavage in our system center on the kinetics of Bid cleavage. Specifically, although PDT caused a rapid disruption of the lysosomes, tBid was not observed until 2 or 3 h post irradiation. There are two possible explanations for the delayed kinetics of Bid cleavage. First, a variety of lysosomal protease inhibitors are normally present in the cytoplasm.[29,40] The enzymatic activity responsible for Bid cleavage may be suppressed by these inhibitors. In essence, the delayed kinetics may simply represent limited availability of active protease. Alternatively, Desagher *et al*.[41] reported recently that Bid is a phosphoprotein, and that the phosphorylated form is resistant to caspase-8-mediated *in vitro* cleavage. Furthermore, *in vivo* studies comparing Fas-induced cleavage of wild-type phosphorylated and mutant non-phosphorylated Bid in engineered cell lines showed that phosphorylation delayed caspase-8-mediated Bid cleavage by at least 1 h.[41] We are currently attempting to determine whether the delayed Bid cleavage seen in out PDT protocols reflects either of the above two situations.

The identity of the lysosomal protease(s) responsible for Bid cleavage is not known. Pharmacological inhibitors of cathepsins B, D and L have implicated initiating and executionary roles for these lysosomal proteases in several apoptotic models.[33,34] However, pharmacological inhibition of these three proteases did not suppress Bid cleavage or pro-caspase-3 activation in our PDT model. Lysosomes/endosomes contain several cysteine proteases besides cathepsins B and L.[29] Stoka *et al*.[19] recently reported that *in vitro* supplementation of lysosomal extracts with the broad spectrum cysteine protease inhibitor E64 also failed to inhibit Bid cleavage. Hence, it is unlikely that lysosomal cysteine proteases are involved in Bid cleavage. We have

identified cell lines which lack the protease responsible for Bid cleavage, and do not undergo apoptosis following PDT with NPe6 (Reiners and Caruso, unpublished data). These lines should aid in the identification of the Bid-cleaving protease.

In our PDT protocol the appearance of tBid preceded/coincided with the release of cytochrome *c* and the activations of pro-caspases-9 and -3. Furthermore, cytochrome *c* release/caspase-9 and -3 activation occurred prior to the loss of $\Delta\Psi_m$. These properties are characteristic of tBid-mediated cytochrome c release.[22–24] We hypothesize that PDT protocols employing lysosomal sensitizers initiate apoptosis by disrupting lysosomes and releasing one or more proteases into the cytosol which are involved in the conversion of Bid to tBid. In turn, tBid triggers the release of cytochrome *c* which stimulates the activation of pro-caspase-3 via activation of Apaf-1/pro-caspase-9. In essence, the generation of tBid by lysosomal enzymes provides a mechanism for how lysosomal disruption can trigger apoptosis via the cytochrome c/Apaf-1/caspase-9 pathway.

Our studies raise two related issues. First, how common is lysosome disruption? Second, how often is cytochrome *c* release and activation of the Apaf-1/caspase-9 pathway actually initiated by lysosome disruption? We do not have the answers to these questions. However, a variety of cytotoxic agents cause lysosomal damage. For example, lysosomal disruption and release of lysosomal proteases into the cytoplasm occurs during alpha-tocopheryl succinate-induced apoptosis in Jurkat T cells,[42] during serum-withdrawal-induced apoptosis in PC12 cells,[30] during naphthazarine-induced apoptosis of neonatal cardiomyocytes[31] and foreskin fibroblasts,[32] and following exposure of macrophages to O-methyl-serine dodecylamide hydrochloride.[43] In at least three of these studies lysosomal damage occurred prior to the release of cytochrome *c* and/or loss of $\Delta\Psi_m$.[32,42,43] Two groups have also reported that H_2O_2 causes lysosome disruption and the induction of apoptosis, necrosis, or both.[44,45] The mode of death appears to be dependent upon the concentration of H_2O_2 used to treat the cells.[44] In H_2O_2-induced apoptosis, lysosome disruption occurred quickly[44] and preceded cytochrome *c* release and loss of $\Delta\Psi_m$.[45] A recent study by Nilsson *et al*.[46] is particularly germane to the questions raised above. Specifically, these investigators demonstrated that lysosomal membranes are very susceptible to damage and disruption by a variety of oxidants. Similar findings have been reported by Brunk and Svensson.[47] It should be noted that oxidants are the cytotoxic species generated in PDT protocols and with naphthazarine. These latter papers, in conjunction with the current study, raise the issue of whether oxidant-induced cytochrome *c* release and Apaf-1/pro-caspase-9 activation are actually the downstream consequences of lysosomal damage. This is a question that we are actively pursuing.

A distinct advantage of the PDT model used in the current study is the specificity with which one can damage lysosomes. The studies reported in Figure 1 clearly indicate that NPe6 preferentially accumulates in a subpopulation of 1c1c7 organelles stained with LTB. An electron microscopy study has also shown that lysosomes are specifically destroyed in NPe6 sensitized, irradiated cells.[10] Although Stoka *et al*.[19] have shown that a lysosomal extract can activate Bid *in vitro*, the current investigation is the first we know of to: (1) demonstrate that targeted lysosome disruption *in vivo* is associated with Bid cleavage, and (2) provide a mechanistic basis for how lysosomal sensitizers in PDT protocols cause cytochrome *c* release and pro-caspases-9/-3 activation. Given the susceptibility of lysosomes to oxidant-induced injury, it is conceivable that lysosomal disruption may be a relatively common initiator of the mitochondrial/intrinsic apoptotic pathway.

Materials and Methods

Chemicals

NPe6 was provided by Dr. Kevin M Smith, Department of Chemistry, University of California at Davis. Fluorescent probes for mitochondrial membrane potential (TMRM), lysosomal integrity (AO or LTB), and chromatin condensation (HO342) were purchased from Molecular Probes (Eugene, OR, USA). Ac-DEVD-AMC and Ac-IETD-AFC were purchased from BD Transduction Laboratories (San Diego, CA, USA). 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide was purchased from Sigma (St. Louis, MO, USA). Z-FA-FMK was obtained from Enzyme Systems Products (Livermore, CA, USA). Pepstatin A, AMC and AFC were purchased from Calbiochem (La Jolla, CA, USA).

Cell culture

Murine hepatoma Hepa 1c1c7 cells were obtained from Dr. JP Whitlock Jr, Stanford University, CA, USA. They were grown in α-MEM supplemented with 5% fetal calf serum and antibiotics in a 5% CO_2 atmosphere, at 37°C, in either culture dishes or on 12 mm glass coverslips coated with poly-L-lysine.

Fluorescent detection of NPe6

Subconfluent monolayers of 1c1c7 cells grown on coverslips were preloaded with NPe6 for ~30 min. After loading the coverslips were washed 3× with PBS and transferred to culture dishes containing fresh medium and 50 nM LTB. After a 10 min incubation at 37°C in a humidified 5% CO_2 chamber, the coverslips were washed 3× with PBS and viewed with a Nikon E600 fluorescence microscope equipped with a Photometrics SenSys CCD camera (Roper Scientific, Trenton, NJ, USA) cooled to −40°C. NPe6 fluorescence was acquired first and was detected using 400–440 nm excitation and 650–700 nm emission. The wavelengths for acquiring LTB fluorescence in the same cultures were 400–440 nm excitation and 450–500 nm emission. After image capture, MetaMorph software (Universal Imaging Corp. Westchester, PA, USA) was used to assign red for NPe6 fluorescence, and green for LTB fluorescence, so that colocalized fluorescent molecules yielded orange/yellow fluorescence.

PDT protocols

Subconfluent monolayers of 2–3 day old cultures were preloaded with NPe6 for ~45 min. After loading the cultures were washed 3× with PBS, refed and then irradiated. Cultures loaded with NPe6 were irradiated for varying lengths of time at 22°C using a 600 W quartz-halogen lamp with IR radiation attenuated by a 10 cm layer of water

and 850 nm cutoff filter. The bandwidth was further confined to 650–700 nm by a broadband interference filter. Light intensity was 1.5 mW/cm^2. Hence, 1 s of irradiation=1.5 mJ/cm^2.

Fluorescence microscopy of mitochondria, lysosomes and nuclei

Apoptotic nuclear morphology was assessed by labeling adherent cells for 10 min at 37°C with HO342 (5 μM). Cultures were then washed 3× with PBS and observed by fluorescence microscopy using 330–380 nm excitation and measuring fluorescence at 420–450 nm. The same cultures were simultaneously loaded with either 0.5 μM AO or 5 μM TMRM in order to image lysosomes or $\Delta\Psi_m$, respectively. The wavelengths for acquiring AO fluorescence were 400–440 nm excitation and 590–650 nm emission. An exposure time of 0.1 s was used to acquire AO fluorescence, and was insufficient to cause AO-mediated lysosome disruption. The wavelengths for acquiring TMRM fluorescence were 510–560 nm excitation and 590–650 nm emission.

Effects of PDT on cell viability

Subconfluent cultures were trypsinized, washed and subsequently suspended in culture medium and plated. NPe6 was added ~16–18 h after plating. After ~45 min the cultures were washed 3× with PBS, and refed immediately prior to irradiation. After irradiation cultures were returned to a humidified 5% CO_2 chamber and incubated at 37°C. The medium was changed every 3 days and colonies were scored 8–10 days after plating. Previous studies have shown that only a low percentage of Hepa 1c1c7 cells divide in the first 20 h after passaging, and that this time is sufficient to replenish the glutathione lost from the cells as a consequence of trypsinization.[48]

Caspases-3 and -8 assays

Cultures were washed twice with PBS before being flooded with lysis buffer (10 mM Tris, pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM sodium phosphate, and 10 mM sodium pyrophosphate). Cells in culture medium and PBS washes were pooled, washed with PBS, and pelleted by centrifugation. After ~3–10 min of incubation on ice, culture dishes were scraped and the lysate was added to the cell pellet derived from the culture medium. The lysate was then transferred to a small tube, sonicated for 1 s, and centrifuged at 13 000 × *g* for 5 min. Supernatant fluids were aliquotted and stored at −80°C. The procedure for assay of caspase-3 using Ac-DEVD-AMC as substrate has been described in detail.[49] Assays for caspase-8/granzyme B activities used Z-IETD-AFC as the substrate and monitored the release of AFC using excitation and emission wavelengths of 400 and 505 nm, respectively. The assay mixture contained 100 mM HEPES (pH 7.5), 10% sucrose, 2 mM DTT, 0.5 mM EDTA and 20 μM Z-IETD-AFC. The assay was initiated by the addition of cell lysate. Release of AMC and AFC was monitored at 37°C with a fluorescence plate reader. Changes in fluorescence over time were converted into pmol of product by comparison to standard curves made with AMC or AFC. Caspases-3 and -8 specific activities are reported as nmol product/min/mg protein. The Bio-Rad Protein assay, using BSA as a standard, was used to estimate protein concentrations.

β-Hexosaminidase assay

The assay described by Storrie and Madden[50] was modified slightly and used to assay β-hexosaminidase. Assays were performed in 200 μl reaction volumes and contained 100 μM sodium acetate buffer (pH 4.4), extract, and the substrate 1 mM 4-methylumbelliferyl N-acetyl-β-D-glucosaminide. Reactions were initiated by the addition of substrate. Substrate cleavage was monitored with a SPECTRAmax Gemini Dual-Scanning Microplate Spectrofluorometer using excitation and emission wavelengths of 364 and 448 nm, respectively. β-Hexosaminidase activity is reported as relative fluorescence units (RFU)/min/μg protein.

Preparation of cytosol for Bid cleavage assays

Cells were released from culture dishes with trypsin/EDTA, mixed with αMEM plus 5% FBS, and pelleted by centrifugation. The pellet (~10^8 cells) was resuspended in PBS. The cell suspension was pelleted by centrifugation and resuspended in 0.25 M sucrose. After an additional washing with 0.25 M sucrose the cell pellet was resuspended in 1 ml of 0.25 M sucrose and homogenized with a dounce tissue grinder (Wheaton #357538) using 15 strokes. The resulting homogenate was sequentially centrifuged at 14 000 × *g* for 5 min and 100 000 × *g* for 1 h. The supernatant fluid represented 'cytosol' and was aliquotted and stored at −80°C.

Preparation of lysosomes and mitochondria

The procedure described by Storrie and Madden[50] was modified in order to purify lysosomes and mitochondria from 1c1c7 cells. Cultures (20 × 100 mm culture dishes) were washed twice with PBS and once with 0.25 M sucrose before the addition of 200 μl/100 mm culture dish of 0.25 M sucrose. The cells were scraped from the plates, combined and homogenized with a motor-driven teflon pestle and plastic-coated tissue grinder (Wheaton #358005) using 4 strokes. The homogenate was centrifuged at 1300 × *g* for 5 min. The pellet was resuspended in 1 ml of 0.25 M sucrose, and recentrifuged as before. The combined supernatant fluids were layered on a density gradient consisting of 35% metrizamide, 17% metrizamide, 6% Percoll (all dilutions were made in 0.25 M sucrose) and centrifuged at 56 000 × *g* for 15 min. To isolate lysosomes 800 μl of the Percoll/17% metrizamide interface was removed and combined with 800 μl of 70% metrizamide and overlayed with 17% metrizamide, 5% metrizamide and 0.25 M sucrose. This gradient was centrifuged as before. Lysosomes banding at the 5%/17% metrizamide interface and mitochondria banding at the 17%/35% interface of the first density gradient, were diluted to 24 ml with 0.25 M sucrose and centrifuged at 56 000 × *g* for 20 min. The lysosomal and mitochondrial pellets were resuspended in lysis buffer (see caspase assays), sonicated for 1 s, and centrifuged at 14 000 × *g* for 10 min. Supernatant fluids were aliquotted and stored at −80°C.

In vitro Bid cleavage assay

Cytosol (20 μg) was incubated with or without 2.5 μg of lysosomal extract for either 30 or 120 min at 37°C in a 30 μl reaction mixture containing 100 μM sodium acetate, pH 5.5. Reactions were terminated by the addition of SDS–PAGE loading buffer.

Western blot analyses

A procedure described by Fan *et al*.[51] was used to analyze for mitochondrial cytochrome *c* release. Cultures were released by trypsin/EDTA treatment and pelleted by centrifugation. The pellets were washed once with ice-cold PBS and resuspended in five volumes of homogenization buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and 250 mM sucrose). After incubation

on ice for 15 min the cell suspensions were homogenized with 15 strokes of a tightly fitting, non ground glass Wheaton Dounce homogenizer. The homogenate was successively centrifuged at $750 \times g$ for 10 min, $10\,000 \times g$ for 15 min, and then $100\,000 \times g$ for 1 h. All centrifugations were performed at 4°C. The $10\,000 \times g$ pellet was used as a source of mitochondria. The $100\,000 \times g$ supernatant fluid represented the cytosolic fraction. Mitochondrial pellets and supernatant fluids were frozen and stored at −80°C until used.

Polypeptides in cytosolic supernatant fluids, or extracts from purified mitochondria or lysosomes, were separated on 12.5% (for caspase-9 and cathepsin D) or 15% (for Bid and cytochrome *c*) polyacrylamide-SDS gels, and electrophoretically transferred onto nitrocellulose. After transfer the blots were incubated with blocking solution (5% Carnation dehydrated nonfat milk in PBS/0.1% Tween 20) overnight at 4°C. Blocked blots were washed with PBS/0.1% Tween 20 and subsequently incubated overnight at room temperature with a monoclonal antibody made to pigeon cytochrome *c* (PharMingen, San Diego, CA, USA), or a rabbit polyclonal antibody made to murine caspase-9 (product #9504; Cell Signaling Technology, Beverly, MA, USA), or a rabbit polyclonal antibody made to recombinant DNA-derived murine Bid[21] or a rabbit polyclonal antibody made to human Cathepsin D (product Im16, Oncogene Research Products, Boston, MA, USA). All antibodies were diluted in PBS, 0.1%Tween-20, 5% BSA. Primary murine antibodies were detected with sheep anti-mouse IgG conjugated with horseradish peroxidase (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA). Primary rabbit antibodies were detected with horseradish peroxidase-linked donkey anti-rabbit IgG (Amersham Pharmacia Biotech, Inc.). Immune complexes were visualized with an ECL detection kit (Amersham Pharmacia Biotech, Inc.) and recorded on X-ray film.

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